Characterization of the Nitrate Reductase Systems in *Salmonella enterica* serovar Typhimurium during the course of the infection process in the mammalian gastrointestinal tract

Karen Prior BSc PGCE(M)

Thesis for the degree of Doctor of Philosophy

Institute of Food Research and
School of Biological Sciences,
University of East Anglia

December 2011

Acknowledgements

My first thanks go to my supervisors, Jay Hinton early on in my work, more recently, Gary Rowley, and throughout, David Richardson. In particular, Gary's unwavering support and help, having been drafted unexpectedly into my supervisory team, have been invaluable. David's continued involvement, support and voice of reason have ensured an element of constancy throughout the last four years. Individuals at the Institute of Food Research have also offered help and support; Isabelle Hautefort, who initiated me into laboratory life, Vitty, Sacha, Steve and Chris to name a few. Corinne first gave help and support at the Institute of Food Research, and later also at the University of East Anglia. Involvement in two labs at UEA has been fantastic, with assistance always freely offered by fellow students and postdocs Heather, Daniela, Andy, Anke, Felicity, Dave, Matt, Matt, Joerg and my favourite nemesis, Ryan. Elaine, what would I have done without you, too, with your brilliant technical support? Thank you all so much.

Family and Friends have also been unstinting in their support. Bob, Hannah and Eleanor, thank you for being so supportive and putting up with some very unusual working hours, and the odd temper tantrum. My girls have grown into beautiful and talented young women in their own right, and about to spread their wings too, while I have been on my own journey of discovery. Never stop learning, my Darlings. All my love and thanks, always. My Mum, for constantly giving me the benefit of your advice, even though things haven't been easy for you in recent years, and listening when I had to offload during some sticky patches. Love you, Mum. Thanks always to Rachel, my undergraduate study buddy and more recently, confidante and counsellor, herself embarking on a brilliant teaching career yet always finding time for me too. Forever friends.

I couldn't have done it without any of you.

Abstract

Salmonella and other pathogens have evolved systems to avoid the innate mammalian immune response. Little is known about how *Salmonella* survive the mammalian gastrointestinal tract during early infection and aspects of *Salmonella* metabolism in relation to virulence have yet to be studied, despite earlier work on virulence determinants (Darwin and Miller 1999; Wallis and Galyov 2000; Zhang, Kingsley et al. 2003).

Since the gastrointestinal tract is largely anaerobic with microaerobic niches (Jones, Chowdhury et al. 2007), nitrate can act as a terminal electron acceptor (Lundberg, Weitzberg et al. 2004). In conjunction with this, previous data (Alston *et al.*) found that SPI-1 genes, required for invasion of epithelial cells, are co-expressed with genes of the *nap* operon. We hypothesised that the *nap* operon is important in the early stages of *Salmonella* infection and postulated that bacterial infection, colonisation of the mammalian gastrointestinal tract and nitrate dissimilation are connected

In this study we elucidate the role of the three nitrate reductase systems of *Salmonella*, NR-A, Nap and NR-Z, during infection. We also characterised the role of NO detoxification by NorV, NrfA and HmpA in the growth and/or survival of *Salmonella* in the gastrointestinal tract. This project provides the first integrated view of the importance of nitrate reduction on gut colonization by *Salmonella*.

Table of Contents

List of Figures

List of Tables

List of Equations

Abbreviations

ATR	Acid Tolerance Response
cat	chloramphenicol
cfu	Colony Forming Units
CI	Competitive Index
℃	Degrees Centigrade
CRP	cAMP Receptor Protein
DNA	Deoxyribonucleic Acid
eNOS	Endothelial Nitric Oxide Synthase
Fis	Factor for Inversion Stimulation
FLP	Flippase Recombinase
Fur	Ferric Uptake Regulator
g	Grams
HilA	Hyperinvasion Locus Protein A (transcriptional activator)
H-NS	DNA Binding Nucleoid Associated Protein
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal
kan	Kanamycin
LB	Luria-Bertoni
µg	Micrograms

µl	Microlitres
µM	Micromolar
mg	Milligrams
ml	Millilitres
mM	Millimolar
M	Molar
NAD+	Nicotinic Adenine Dinucleotide
NADH	Nicotinic Adenine Dinucleotide (reduced)
NAD(P)+	Nicotinic Adenine Dinucleotide Phosphate
NAD(P)H	Nicotinic Adenine Dinucleotide Phosphate (reduced)
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNI	Reactive Nitrogen Intermediates
RpoE	RNA Polymerase Subunit E (Alternative Sigma Factor)
RpoS	RNA Polymerase Subunit S (Alternative Sigma Factor)
rpm	Revolutions Per Minute
RVR	Relative Virulence Ratio
SCV	*Salmonella* Containing Vacuole
SPI1	*Salmonella* Pathogenicity Island 1
SPI2	*Salmonella* Pathogenicity Island 2
GSNO	*s*-Nitrosoglutathione
TTSS	Type Three Secretion System
UQ	Ubiquinone
UQH_2	Ubiquinone (reduced)
V	Volts

Chapter 1 – Introduction

1.1 The *Salmonella* story

The *Salmonella* bacterium was isolated in 1885 by a veterinary surgeon, D.E.Salmon, and his research assistant, T.Smith, himself a respected epidemiologist and pathologist, in the course of their work on pigs. The disease which it was thought to cause at that time was known as 'hog-cholera', so they named this Gram negative bacillus Hog-cholerabacillus. Later, Joseph Léon Marcel Ligniéres honoured Salmon by re-naming the bacteria *Salmonella choleraesuis.* This bacterium ultimately turned out to be commensally present in pigs, the causative agent of hog cholera being a virus. We now recognise two discrete species of *Salmonella*, *Salmonella enterica* (*S. enterica*) *and Salmonella bongori* (*S. bongori*); all of the different serological variants (serovars, or sv.), of which there are now over 2500, fall into one of these two species.

1.1.1 Classification

Classification of species has variously been based on factors such as host specificity, susceptibility to phage and the presence of cell surface antigens like lipopolysaccharide (LPS). The different serovars of *Salmonella* are defined by the different combinations of O, H1 and H2 antigens present on their bacterial cell surface. In 1952, Edwards and Kaufmann developed a method for classification in which flagellar (H) antigens of two different types (H1 – monophasic and H2 – diphasic) were used (Edwards and Kauffmann 1952). Depending on the oligosaccharide arrangement, there are also 50 different types of LPS antigen which can be identified. Subsequently, Kauffmann further refined the system by including classification based on cell surface LPS antigens (O or somatic antigen). Consequently, the Kauffmann-White schema

now applies the rule that each discrete combination of O, H1 and H2 identifies a different serovar. Further work has produced new proposals for improvements to the Kauffmann-White schema (Le Minor, Veron et al. 1982; Le Minor and Bockemuhl 1988), with the resulting identification of further serovars (Popoff, Bockemuhl et al. 2003; Popoff, Bockemuhl et al. 2004). Names given to the serovars often indicate the host and disease symptoms, such as with *Salmonella choleraesuis* explained earlier, or where they were first identified, for example *Salmonella dublin*.

Use of DNA hybridisation showed that all the *Salmonella* serovars have a single hybridization group, so form a single species comprising seven subspecies (Crosa, Brenner et al. 1973; Stoleru, Le Minor et al. 1976). The species name *Salmonella enterica* (*S. enterica*)(sub-species (I))is now further defined by *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *bongori* (V) and *indica* (VI). Still, the common serovar names, such as '*Salmonella enterica* serovar Typhimurium' (*S.* Typhimurium) have been kept in use for subspecies (I) strains. 60% of all serotypes reside in subspecies (I), and over 90% of those cause human salmonellosis (Neidhardt 1996).

Salmonella classification remains extremely complex. The use now of genome based identification technology has negated the need for earlier methods. Genome sequencing has importantly revealed that two pathogenically important variants are so closely related in their DNA that they cannot be considered separate species. These are *S*.Typhimurium LT2 (McClelland, Sanderson et al. 2001) and *Salmonella* Typhi (*S.* Typhi) (Parkhill, Dougan et al. 2001), which have up to 99% sequence homology (Edwards, Olsen et al. 2002). This has led to the current classification of only two *Salmonella* species, *Salmonella enterica*, which contains former subspecies I, II, IIIa, IIIb, IV, and VI, and *Salmonella bongori* (formerly subspecies (V) (Selander, Beltran et al. 1990; Boyd, Wang et al. 1996; Chan, Baker et al. 2003).

Most members of the *Salmonella* genus are fermentative facultatively anaerobic intracellular pathogens of humans, birds and/or animals, and although there is a high degree of similarity in DNA between different serovars, they infect a variety of hosts to different extents. Most serovars are also highly motile, carrying peritrichous flagella distributed over their entire surface. As emphasised here earlier, most of the pathogenic variants belong to the *Salmonella enterica* species, including Typhimurium, Typhi, Paratyphi and Enteritidis serovars, although the degree of host speciation affects the type and levels of pathogenicity displayed in different hosts; for example, while *S.* Typhimurium causes a serious systemic infection in mice, it is more likely to result in a troublesome, though relatively minor, infection of the gastrointestinal tract in humans.

1.1.2 *Salmonella* physiology

The Salmonellae are classified as γ-proteobacteria, confirmed by their ribosomal RNA sequences, and included in the taxonomic family Enterobacteriacae (Cohen, Mechanda et al. 1996; Bohnert, Hubner et al. 2000; Lupp, Robertson et al. 2007).As a Gram negative bacterium, *Salmonella* is surrounded by a complex membrane structure. Around the plasma membrane is an additional thin layer of peptidoglycan which is itself surrounded by an outer membrane covalently attached to the peptidoglycan layer by lipoproteins. The outer membrane houses the O, H1 and H2 antigens which dictate the serological differences between serovars. Since they are facultative anaerobes, they are able to survive and grow either aerobically or anaerobically (Neidhardt 1996). In addition, a high level of motility may aid the ability of the bacterium to evade

some host defences (Stecher, Barthel et al. 2008), making *Salmonella* a formidable pathogen in a wide range of species.

1.1.3 *Salmonella enterica* epidemiology

There were 9,079 human infections reported in England and Wales during 2009 which were attributed to *Salmonella* (HPA 2010). This number is probably under-reported by a considerable margin, due to the self-limiting nature of most *Salmonella* infections (Oosterom 1991). It has also been estimated that there are between 16 million and 33 million cases of Typhoid Fever per year around the World (DeRoeck, Jodar et al. 2007), and overall, *Salmonella* infections kill around 1 million people each year (Galanis, Lo Fo Wong et al. 2006; Hendriksen, Mikoleit et al. 2009). However, the epidemiology of salmonellosis infection is difficult to measure precisely, due to differences in disease recording and presentation of patients between countries. Often, those areas most affected, such as south-central and south-east Asia, Africa, the Caribbean, and Oceania, also have the least effective recording systems for infectious diseases (Crump, Luby et al. 2004). Typhoid Fever is rare in Europe, but widespread in other parts of the World where water sanitation is less efficient. In sub-Saharan Africa and Asia in particular, prevalence of typhoidal salmonellosis is high, mainly due to poor sanitation; the death rate is also very high, since medical intervention may be difficult to access. The annual frequency has been estimated to be around twenty-seven million new cases of Typhoid Fever worldwide (Crump, Luby et al. 2004).

1.1.4 Potential sources of infection

Infection of humans by *Salmonella* often arises from consumption of contaminated food as well as water, although direct human to human, and animal to human transmission can occur. In this sense, *Salmonella* infections can be considered zoonotic. The most common food sources of *Salmonella* are poultry and egg products; beef and pork have also been found to be a *Salmonella* reservoir (Adak, Meakins et al. 2005).Indeed, in 2003, *Salmonella* was isolated from the caeca of 23.4% of British pigs in a survey carried out by D.E.F.R.A. The most common serotype found in pigs was *S*. Typhimurium, also often associated with human infections. Pork is also the most consumed meat in Europe (Fosse, Seegers et al. 2008). Of even more concern perhaps is the finding that in the United States, 90% of the *Salmonella* strains isolated from pig carcasses are multidrug resistant (Hoelzer, Soyer et al. 2010). In contrast, only 1.4% of cattle carcasses carried predominantly *S.* Dublin, which itself only rarely causes human infection. In sheep *S*. Enterica subspecies *diarizonae* appeared with highest frequency, though still in only 1.1% of carcasses (D.E.F.R.A. 2005). The European Food Safety Authority also concluded that the lymph nodes of 21% of British pigs were infected with *Salmonella*, (13.8% *S*. Typhimurium and 4.8% *S*. Derby) (E.F.S.A. 2008).

In contrast to the picture for *Salmonella* in the United Kingdom food production industry, *Campylobacter* species were discovered in 54.6% of cattle, 43.8% of sheep and 69.3% of pigs at slaughter. *C. jejuni* accounted for 81% and 65% of the *Campylobacter* presence in cattle and sheep respectively. Interestingly, 90% of the *Campylobacter* isolated from pigs was *C. coli* (D.E.F.R.A. 2005).*Campylobacter* infections are responsible for most of the instances of human food poisoning reported in the United Kingdom, although it is *C. jejuni* that causes most cases of infection (Tam, O'Brien et al. 2003); this suggests

that most infections may not originate from the ingestion of pork products. Still, cases presenting with *C*. *coli* infection account for about four times more cases of indigenously acquired food borne disease (IFD) than those arising from *S.* Typhimurium infection. However, *S.* Typhimurium is responsible for more human deaths (Tam, O'Brien et al. 2003). Verotoxigenic *Escherichia coli* (VTEC 0157) was present at much lower levels, 4.7% in cattle, 0.7% in sheep and only 0.3% in pigs. Verotoxin, also referred to as Shiga-like toxin, is one of a class of toxins that inhibit protein synthesis by blocking the interaction of ribosomal RNA. This toxin is generated by some strains of *Escherichia coli*. It is named for its similarity to the AB5-typeShiga toxin produced by the bacteria *Shigella dysenteriae*. A variety of shiga-like toxins are produced by pathogenic strains of *E. coli*, in particular *E. coli* O157. The toxins are particularly interesting since they require highly specific receptors on the cells' surface in order to attach and enter the cell; species such as cattle, swine and deer which do not carry these receptors may harbour toxigenic bacteria without any ill effect, shedding them in their faeces, from where they may be spread to humans.

In December 2007, the United States Department of Agriculture was forced to issue a public health warning when 38 people became ill after eating ground beef, apparently purchased from a supermarket chain (CIDRAP 2007). An additional concern was that the strain involved proved to be the multi-drug resistant *S.* Newport, with the – MDR-AmpC phenotype. This incident serves to show that *Salmonella* infection of humans is not restricted to the developing world, and may become increasingly difficult to deal with effectively, as levels of antibiotic resistance increase. In January 2006, the United Kingdom-based chocolate manufacturer Cadbury withdrew many of its products when about 60 cases of salmonellosis were traced to the ingestion of Cadbury products. The problem was identified as a leaking pipe which had become contaminated with

Salmonella at a factory in Herefordshire. It was estimated that the cost of this error amounted to some £10M to the company. Fruit and vegetables also provide a reservoir for infection (Adak, Meakins et al. 2005) and in June 2008, the United States Food and Drug Administration (FDA) warned of a serious outbreak of salmonellosis initially connected to raw tomatoes. Subsequently, jalapeño peppers were found to be the culprits, probably as a result of the plants being watered with contaminated water. According to the Centre for Disease Control (C.D.C.), a total of around 1500 confirmed cases of salmonellosis were attributed to this outbreak (C.D.C 2008). In January 2009, an outbreak of *S*. Typhimurium infection in the United States was traced to a factory making peanut butter, and owned by the Peanut Corporation of America (PCA). The FDA recommended avoidance of commercially-prepared or manufactured peanut butter-containing products and institutionally-served peanut butter. *S*. Typhimurium was identified in peanut-butter based products as diverse as crackers, energy bars, ice cream and cookies across 46 states in the US, which had been manufactured by over 300 different food companies. Around 700 people became ill as a result, and nine deaths were attributed to this outbreak over the next three months. The peanut butter and peanut paste manufactured by PCA had been distributed to hundreds of other food producing companies for use as an ingredient in many different products, all of which were recalled. Outbreaks of this nature, where an ingredient which may be used in many different products, present an extra challenge in terms of detection, tracing and limiting the potential damage which may arise (Cavallaro, Date et al. 2011).

The CDC also later reported another outbreak associated with ingestion of raw vegetables, this time alfalfa sprouts, (C.D.C. 2009). In fact, it is thought in this outbreak, it was a batch of the alfalfa seeds which had become contaminated from the seed grower. Thus, the economic costs and impact potential of *Salmonella* should not be underestimated. Recent assessment in the United

States concluded that the annual costs associated with *Salmonella* infections there amounts to an estimated $2.65Billion (CIDRAP 2010).

Foodborne *Salmonella* infections are not the only source; as previously alluded to, zoonotic transmission can also occur and this commonly happens in households where reptiles of various species are kept as pets. The *Salmonella* serovars may vary, but the disease outcome, an episode of acute enteritic symptoms, is characteristic (Sanyal, Douglas et al. 1997). This work found evidence of two different *Salmonella* strains in one household in which two different reptilian species were kept, *Salmonella chameleon* in an iguana, and *Salmonella arizonae* in four snakes.

1.1.5 Host specificity and Types of Disease

Salmonella serovars may be either host specific, in which case they are restricted to infection of only one specific host species, host adapted which allows for the infection of a few different species, or generalists, which infect a wide range of hosts (Maskell 2005).However, the type of disease and symptoms vary between host species, even where the same serovar is involved. An example of such variation arises in infections caused by *S*.Typhimurium, which is usually limited to causing gastroenteritis infections in humans. In contrast, in mice, *S*. Typhimurium results in a systemic infection similar to Typhoid Fever, ultimately causing the death of the affected animal. These differences have attracted much interest in the pathogenetic attributes of the variants, since identification of the various virulence factors provides targets for either vaccine or antibiotic intervention (Germanier and Furer 1975; Galan and Curtiss 1989; Morgan, Campbell et al. 2004; Becker, Selbach et al. 2006).A host specific example is *Salmonella enterica* serovar Typhi (*S*. Typhi), which causes Typhoid Fever specifically in humans (Meltzer and Schwartz 2010). Chimpanzees may also be a closely enough related species to human for the specificity to also

apply to them, and this is supported by the observation that a disease which closely mimics that in humans can be caused in chimpanzees by challenge with *S*. Typhi (Gaines, Sprinz et al. 1968).

1.1.5.1 Typhoid Fever

*S.*Typhi and *Salmonella enterica* sv. Paratyphi (*S*. Paratyphi) are transmitted in contaminated water and cause Typhoid or Paratyphoid fever in humans respectively. These systemic infections are acquired via the faecal-oral route, and involve organs other than the gastrointestinal tract, in the reticuloendothelial system which includes the liver and spleen. Paratyphoid fever resembles Typhoid Fever but presents with a more abrupt onset, milder symptoms and a shorter course (Bhan, Bahl et al. 2005).

Infections are usually the result of ingesting contaminated water, or food which has been washed using contaminated water (Sears, Ferreccio et al. 1984; Mahle and Levine 1993; Huang and DuPont 2005). *S*. Typhi causes Typhoid Fever, in which the symptoms are wide-ranging and serious. If Typhoid Fever goes untreated, the course of the disease takes four discrete stages over about a month (Azad, Islam et al. 1997). Initially, there is an increase in body temperature, slow heart rate, headache, cough and general malaise (Ivanoff, Levine et al. 1994). Occasionally, there is bleeding from the nose and abdominal pain (Borries 1930). A reduction in the number of white blood cells and eosinophils is observable (Wang, Gu et al. 1989); blood cultures test positive for *S*. Typhi or Paratyphi. The classic Widal test is usually negative in the first week. However, results of this test must be interpreted cautiously; although it is a serological test for enteric or undulent fever, it is not very specific (Pang and Puthucheary 1983; Parry, Hoa et al. 1999). The Widal test uses agglutination of antibodies against O-somatic and H-flagellar antigens in the blood, and as

patients may have been exposed to other species of bacteria which cause cross-reactivity a false-positive result can be indicated (Senewiratne and Senewiratne 1977; Pang and Puthucheary 1983). Also, any past history of enteric fever, typhoid vaccination, and the general level of antibodies in the populations in areas of the World in which typhoidal infections are endemic can influence the result. In the second week, the fever plateaus at around 40 ℃ (104 ℉) (Parry, Hien et al. 2002; Nsutebu, Martins et al. 2003).Delirium frequently occurs, and rose spots appear on the lower chest and abdomen in 30% of cases (Wang, Gu et al. 1989). There are rhonchi (rattling sounds) from the lungs, (Neva 1950) which can lead to an initial misdiagnosis of upper respiratory tract infection. The abdomen may distend and become painful in the right lower quadrant where borborygmi (rumbling noises) can be heard (Morgenstern and Hayes 1991; Hohmann, Oletta *et al*. 1996). Diarrhoea can occur in this stage (more common in children) although constipation (more common in adults) may also do so (Abdool Gaffar, Seedat et al. 1992; Khan, Coovadia *et al*. 1999). Hepatosplenomegaly (enlargement of liver and spleen) develops (Hosoglu, Celen *et al*. 2006). The Widal reaction is by now strongly positive with antiO and antiH antibodies. Complications may begin to manifest during the third week; these can include intestinal haemorrhage, intestinal perforation in the distal ileum (frequently fatal) (Everest, Wain *et al*. 2001; Hosoglu, Aldemir *et al*. 2004; Hosoglu, Celen *et al*. 2006), encephalitis, metastatic abscesses (Gremillon, Geckler *et al*. 1977), cholecystitis (inflammation of the gall bladder), endocarditis(inflammation of the heart valves and/or endocardium) and (rarely) osteitis (bone inflammation) (Allal, Kastler *et al*. 1993; du Plessis, Govendrageloo *et al*. 1997; Malik 2002; Rayan, Mukundan *et al*. 2009).The fever remains very high and dehydration follows. The fever finally reduces after four weeks if the patient survives.

1.1.5.2 Gastroenteritis

Several *Salmonella* serovars cause acute but self-limiting gastroenteritis in humans, primarily sv. Enteriditis and sv. Typhimurium. For *S.* Typhimurium, an incubation period of 6 to 72 hours is followed by varying combinations of headache, abdominal pain, diarrhoea and vomiting. The diarrhoea can contain blood, lymphocytes and mucus. Fever, malaise and muscle aches may also be experienced (Darwin and Miller 1999). In gastroenteritis infections, epithelial cells respond to infection by first increasing their intracellular calcium concentration (Pace, Hayman et al. 1993). This, in combination with the action of specific *Salmonella* virulence factors, activates the transcriptional regulator NF-κB (Rogler, Brand et al. 1998), resulting in the production and secretion of pro-inflammatory cytokines, such as Interleukin 8, a chemo-attractant for polymorphonuclear (PMN) leukocytes such as neutrophils (Elewaut, DiDonato et al. 1999; Gewirtz, Rao et al. 2000). These PMNs migrate to the gut lumen and, in combination with an increased level of epithelial cell death, cause fluid secretion, breakdown and detachment of the epithelial cell layer, which is manifested symptomatically as bloody diarrhoea (Haraga, Ohlson et al. 2008). These aspects of the immune response are covered in more detail later. Most sufferers of *Salmonella*-induced gastroenteritis infection recover within a week; however, the elderly, young children and immuno-compromised individuals may suffer more severe infections, such as a non-typhoidal bacteraemia (Velge, Cloeckaert et al. 2005), and unfortunately some of these victims do not recover. More severe infections may also result if a particular strain of *Salmonella*, such as DT104, is involved.

Gastroenteritis infections caused by *Salmonella* are also typified by the continued faecal shedding of bacteria for up to 18 weeks after the illness was first manifested. Occasionally, some patients become carriers, and despite

being asymptomatic themselves, the faecal shedding of the bacteria continues for periods in excess of one year (Scherer 2001). Carriers present the risk of causing new infections, so their treatment regime must be effective to ensure that further spread of the disease is prevented.

1.2 The biology of *Salmonella* infection

1.2.1 Mammalian Host Gastrointestinal Physiology

It has been suggested that the gastrointestinal system is the largest part of, and the major contributor to, the immune system in the human body (Hart, Lammers et al. 2004).Upon ingestion, the first challenge encountered by enteric pathogens occurs within the oral cavity, where saliva, which contains lysozyme, is produced by the salivary glands. Lysozyme threatens the integrity of many bacterial membranes; it is a bacteriolytic enzyme present in several animal secretions (Peeters and Vantrappen 1975; Jones and Bevins 1992) .

Having passed to the stomach, the bacteria experience a dramatic change in pH, due to the increasingly acidic environment of the stomach. Here, acidity may become as high as pH1 in the immediate post-prandial period (Levy, Koeppen et al. 2005; Rychlik and Barrow 2005). In addition, the presence of digestive enzymes and compounds, intended to break down whatever may be in the stomach, would contribute to the defence system of the host. If the bacterial defences fail, host proteases and peptidases could attack their proteins, reducing them to their component amino acids, carbohydrases break down starch and sugars, such as those found in bacterial membranes, while nucleases will reduce bacterial DNA to base nucleotides.

Upon leaving the stomach via the pyloric sphincter, the gut contents are met by a much more alkaline environment in the small intestine. Here, pancreatic juices and bile salts are secreted into the gut lumen, ensuring that the small intestine is maintained at an alkaline pH, (Levy, Koeppen et al. 2005), which prevents physiological damage to this part of the gastrointestinal tract and also allows for correct absorption of nutrients, vitamins and minerals which would be disrupted by an acidic environment.

The epithelial surface of the mammalian small intestine comprises mainly columnar absorptive enterocytes, interspersed with Paneth cells, and goblet cells (Sansonetti 2004). The enterocytes are polarised cells, with a serosal or baso-lateral surface, and a brush border membrane facing into the lumen. The brush border surface is formed into microvilli which project into the gut lumen. Paneth cells are known to be operational in the immune response, since they secrete lysozyme and defensins to the gastrointestinal lumen as a defence compound (Peeters and Vantrappen 1975; Ganz 2000). Paneth cells are located towards the bases of the crypts of Lieberkühn which are invaginations of the surface of the small intestinal surface (Porter, Bevins et al. 2002). Goblet cells secrete mucus which forms a protective and lubricating barrier around the epithelial surface which is constantly exposed to ‘foreign’ molecules, whether they be food or foe (Deplancke and Gaskins 2001). Work in rats has shown that mucus is present in two layers, the outer one being easily removed, while the inner layer is firmly attached to the epithelium (Johansson, Phillipson et al. 2008). While the outer layer may provide a nutritive resource for some commensal bacteria, the inner layer is impenetrable and prevents contact between the bacteria and the epithelium, (Backhed, Ley et al. 2005), which should also exclude pathogens. This also means that bacteria, whether commensal or pathogenic, are prevented from making contact with cells of the immune system, which could otherwise result in the initiation of an inflammatory

response, including that seen in Salmonellosis, which is evidenced by diarrhoeal symptoms. The physiology of the epithelial surface combined with such an array of defences, in addition to the peristaltic action of the gut and the competition for access to the surface with commensal bacteria which are also present in greater numbers in the gut presents a formidable hurdle to invading pathogens.

Supplementing the barrier effect of the intestinal epithelium, the immune system here is supported further by the presence of gut associated lymphoid tissue (GALT), which comprises the appendix, lymphoid follicles, mesenteric lymph nodes and Peyer's patches. Peyer's patches are organized lymphoid follicles which are located in the wall of the small intestine, and their numbers increase towards the terminal ileum, just prior to the large intestine (Cornes 1965). These structures are dome-shaped follicles covered with mostly columnar epithelial cells. The lack of goblet cells results in a reduced mucus layer over the follicles (Owen and Ermak 1990).However, the Peyer's patches also contain specialized cells called microfold-cells (M-Cells) which are unique to the patches (Bockman and Cooper 1973; Owen and Jones 1974; Jones, Ghori et al. 1994). The M-Cells are adapted from enterocytes and do not have the microvillous brush border membrane which is characteristic of enterocytes (Owen 1994). M-Cells play an important role in the uptake of antigen which then stimulates the mucosal immune response (Wassef, Keren et al. 1989). They achieve this by sampling the microorganisms present in the lumenal environment (Bockman and Cooper 1973; Owen and Jones 1974; Owen and Ermak 1990; Jones, Ghori et al. 1994; Owen 1994). Structurally, M Cells contain a cavity on their serosal side, which is populated by cells of the innate immune system which are ready to pick up microorganisms translocated from the lumen via the M-Cells. By their uptake of bacterial cells for this sampling function, M-cells provide a route through the intestinal epithelial barrier (Zoetendal, Vaughan et al. 2006) which some pathogenic bacteria, like *Salmonella*, can and do, use to their advantage.

It has also been suggested that intestinal dendritic cells (DCs) sample bacteria, such as *Salmonella*, by extending cellular processes into the gut lumen to capture bacteria and draw them back to the serosal side of the epithelium to be dealt with by other killing mechanisms of the immune response (Rescigno, Rotta et al. 2001; Niedergang, Didierlaurent et al. 2004; Niess, Brand et al. 2005). There is again the possibility that these dendritic cells are hijacked by the bacterial cells so that they can infect the host. More recent work has shown that for *Salmonella* which fail to adhere to the epithelium, dendritic cells of the immune system can still sense their presence, either before or perhaps after other *Salmonella* have invaded, and pass through the epithelium to capture them before being ejected from the host via the lumenal route (Arques, Hautefort et al. 2009).

1.2.2 Infection process

The size of the infectious dose required to initiate and then to sustain a *Salmonella* infection depends on the bacterial strain, as well as the physiological state of the host and the types of foods consumed with the bacteria (Darwin and Miller 1999); between a thousand and one million *S.* Typhi bacteria have been identified as sufficient to successfully infect a human host (Hornick 1970; Hornick, Greisman et al. 1970). There are discrete stages of the infection process which occur during following ingestion of an infectious dose via the normal physiological route.

1.2.2.1 Gastrointestinal Phase of Infection

Pathogenic bacteria must be able to evade the defence mechanisms of their host at least for long enough for disease symptoms to appear in their host. If the bacterium is an opportunistic pathogen, it will exploit pre-existing weakness in the host's immune response. However, where the immune system remains

intact, *Salmonella*, together with other enteric pathogens like *Campylobacter*, *Escherichia* and *Listeria,* must first overcome the action of the mammalian host's first immune barrier, the acidic environment which is met in the stomach.

Following ingestion, *Salmonella* switches on an acid-stress response enabling it to survive the acidic environment of the stomach (Foster 1991; Gahan and Hill 1999). This response has been well-characterized, and is an adaptive response to lower pH levels, called the Acid Tolerance Response (ATR). Enteric bacteria principally rely on the global regulator RpoS to control the acid stress response (Foster 2004), although other regulatory proteins PhoPQ and Fur are also involved in the acid stress response in *Salmonella* (Rychlik and Barrow 2005). In *S.* Typhimurium, and *C. jejuni*, the ability to withstand low stomach pH is enhanced by the capacity of these microbes to 'pre-adapt' to an acid environment by employing their ATR (Foster 1991; Murphy, Carroll et al. 2005), and it is induced by exposure to a mildly acidic pH of between 5.5 and 6.0.This may also help to explain the observation that some individuals who habitually use antacid medication are more susceptible to *Salmonella* infections (Smith 2003). After survival in the stomach *Salmonella* pass through to the small intestine, where they experience another change in their environment to one which is more alkaline.

1.2.2.2 Invasion phase of Infection

Penetration of the epithelial cells which line the gastrointestinal tract is vital to the progression of the infection of a host by *Salmonella*. During the initiation of an infection in the small intestine, *Salmonella* must adhere to the brush border membrane surface of the cells lining the gut epithelium (Haque, Bowe et al. 2004; Haraga, Ohlson et al. 2008). In mice, entry of *Salmonella* to the gastrointestinal epithelium occurs only via M-cells within the Peyer's Patches

(Jepson and Clark 2001). The *Salmonella* infection route in calves is via enterocytes or M-cells distributed along the epithelial lining other than the follicle-associated epithelium, with no apparent preference for either route (Wallis and Galyov 2000; Zhang, Kingsley et al. 2003). The precise route in humans is still to be elucidated, though it may be reasonable to conclude that M-cells could provide a major route for entry in the human gut (Jepson and Clark 2001). It has also been shown that on some occasions, *Salmonella* may effect entry via enterocytes (Haque, Bowe et al. 2004). The presence of the bacteria is sensed by the host cells via specific recognition by toll-like receptors (TLRs) of molecular motifs. This recognition by the TLRs of the presence of bacterial threats results in the switching on of signal transduction pathways in the host which lead to the expression of genes which control the innate immune response as well as the development of acquired immunity (Takeda and Akira 2005). TLR4 in particular is responsive to the bacterial lipopolysaccharide (LPS) of Gram negative bacteria (Takeuchi, Hoshino et al. 1999) while TLR5 recognises the molecular motifs present in bacterial flagellin (Fournier, Williams et al. 2009).The molecular motifs are present on the surface of bacterial cells, and known as pathogen-associated molecular patterns (PAMPs) (Mumy and McCormick 2005). The PAMPs enable the host cells to identify the bacteria as 'non-self' cells, and target the foreign agents for attack by the host immune system (Coburn, Grassl et al. 2007). The PAMPs which stimulate the immune system response to *Salmonella* cells include several bacterial cell components such as the LPS component of the bacterial membrane, and flagellin, a protein which polymerises into a hollow cylinder, forming the filament of the bacterial flagellum (Philpott and Girardin 2004; Coburn, Grassl et al. 2007), involved in bacterial motility. It has however been shown that the main PAMP which mediates the response to *Salmonella* is LPS, and consequently, the cascade of responses from the immune system which follows recognition by TLR4 (Roy, Lariviere et al. 2006).

In preparation for invasion into non-phagocytic cells, such as epithelial cells, genes within the tightly regulated *Salmonella* Pathogenicity Island 1 (SPI-1) are induced in response to several inducing environmental cues. Changes in acidity and alkalinity during the transit through the gastrointestinal tract may induce SPI-1 expression, and this is explained in more detail later. SPI-1 induction can also be effected by increasing salt levels and this can be used to experimental advantage when induction is required, such as during invasion assays. Changes in the oxygen levels during gut transit, as well as exposure to metabolites produced by the usual gut flora and response to the presence of antimicrobial peptides produced by the host also alter the microbial environment (Rychlik and Barrow 2005).SPI-1 encodes a hypodermic syringe-like Type-III Secretion System (TTSS), through which *Salmonella* secretes pre-synthesised effector proteins (Geddes, Cruz et al. 2007). TTSS are complex structures which are employed by several pathogenic species to introduce bacterial proteins into host cells (Hueck 1998). The apparatus is assembled from components for which gene expression is conserved between species. The evidence suggests that TTSS have been acquired through horizontal transfer of genes in the course of bacterial evolution (Hueck 1998; Lawrence and Ochman 1998; Wang 2001).

Several effector proteins are required for this step in the infection process orchestrated by *Salmonella* (Galan and Zhou 2000; Geddes, Cruz et al. 2007). SopA (an E3 ligase) (Diao, Zhang et al. 2008) and SopB (an inositol polyphosphate phosphatase) (Galan and Zhou 2000)stimulate proteins within the host cell to effect cytoskeletal rearrangements of the host cell. They achieve this by stimulating Cdc42 and Rac, small GTP binding proteins present in the cell. SipA (*Salmonella* invasion protein) has been shown to lower G-actin concentrations and bind F-Actin, and in doing so, it causes conformational changes to the actin-based cytoskeleton of mammalian cells (Galan and Zhou

2000; Galkin, Orlova et al. 2002). Triggering the Cdc-42 and Rac-1 Rho-GTPase-mediated manipulation of the actin cytoskeleton of the host cell is such that the host cell membrane forms ruffles which surround and engulf the bacterial cell (Chen, Hobbie et al. 1996; Hardt, Chen et al. 1998; McGhie, Brawn et al. 2009). This is reminiscent of the behaviour exhibited by phagocytic cells and results in the internalisation of the *Salmonella* cell to the mammalian host cell (Jepson, Kenny et al. 2001; Hapfelmeier, Ehrbar et al. 2004; Perrett and Jepson 2009). Additional effectors which regulate the actin cytoskeleton and contribute to invasion of cells by *Salmonella* are SopE, SopE2 and SopB (Perrett and Jepson 2009). The processes involved are illustrated in Figure 1.

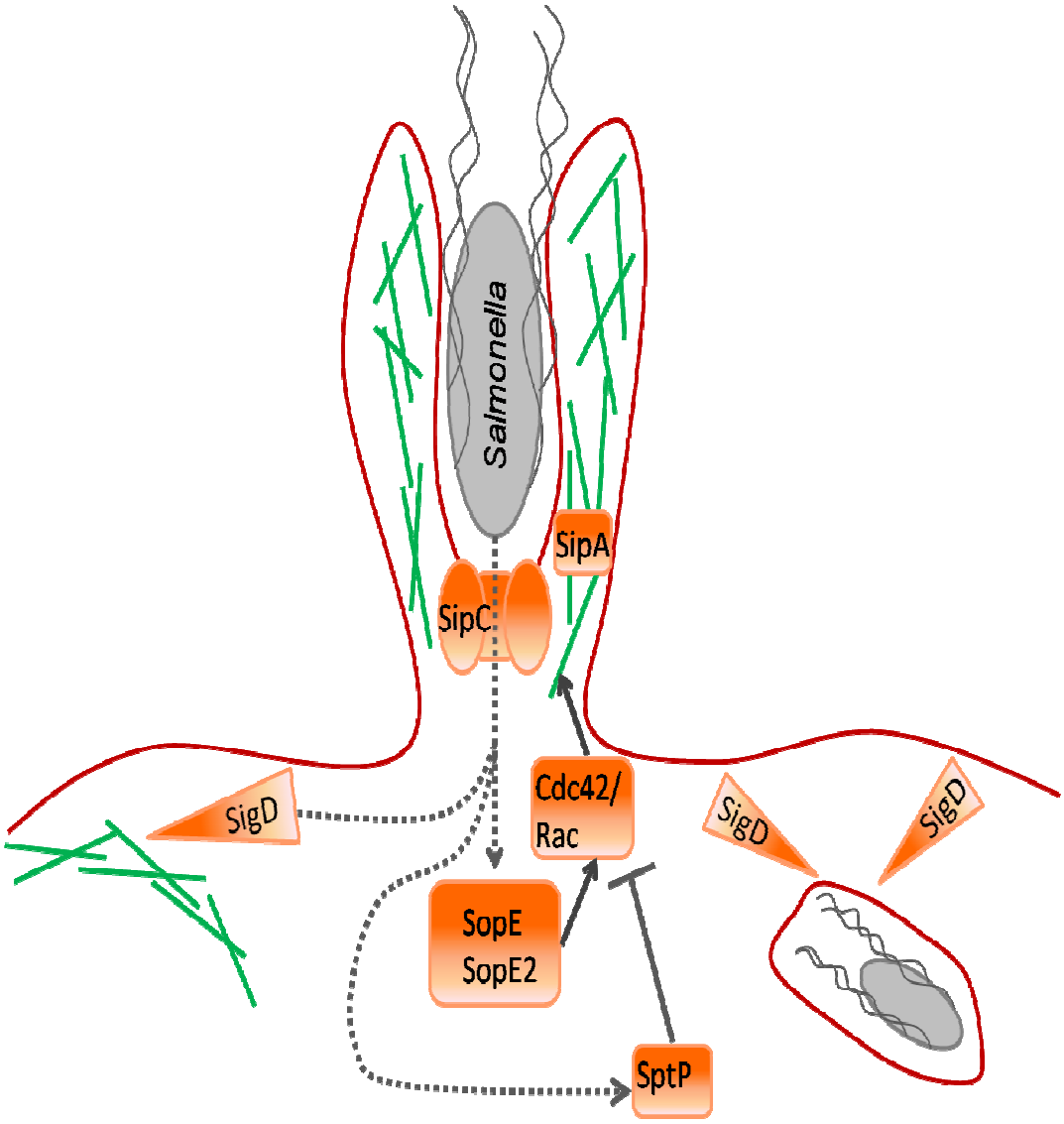


Figure 1 – Invasion of Host cells by *Salmonella* is mediated by the *Salmonella* pathogenicity island-1 (SPI-1) type III secretion system (TTSS) and its effectors. SigD/SopB disrupt the attachment of the cell membrane (red) and actin cytoskeleton (green). SopE and SopE2 enhance Cdc42 and Rac1 activity. SipA and SipC alter cytoskeletal structure, SipC nucleates actin, initiating polymerization and SipA binds actin and alters actin bundling. SptP is a GTPase activating protein which can inhibit the changes in the cytoskeleton, by inactivating Cdc42 and Rac. SigD subverts the cellular cytoskeleton and seals the resultant invaginations of the membrane which ultimately forms the *Salmonella* containing vacuole.

SPI-1 mutants are still able to infect the host following intraperitoneal (IP), infection to the same extent as wild type *Salmonella* (Galan 1996; Ochman, Soncini *et al*. 1996).However, it is interesting that SPI-1 mutants fail to infect the host following oral infection, and also that SPI-1 mutants are not able to invade enterocytes *in vitro* (Lucas and Lee 2001). This suggests that while SPI-1 is required for invasion via the gut epithelium, it is not necessary for intracellular survival and the systemic phase of infection.

The TTSS apparatus is assembled in response to gene expression induced by a range of conditions (Johnston, Pegues et al. 1996)which are present following oral ingestion. The conditions include basic pH (alkalinity, such as is found in the mammalian small intestine), low oxygen and high osmolarity (Ernst, Dombroski et al. 1990; Lee and Falkow 1990; Bajaj, Lucas et al. 1996).Regulation of the TTSS is highly complex. The transcription factor HilA is one participant which activates the expression of genes required for TTSS-1, and this is encoded within SPI-1 (Bajaj, Hwang et al. 1995). Consequently, the ability of *S*. Typhimurium to successfully invade the mammalian epithelium via SPI-1 activation is completely dependent on functional HilA (Bajaj, Hwang et al. 1995; Bajaj, Lucas et al. 1996). HilA itself is activated in part by SirA (*Salmonella* invasion regulator), and the invasion capability of *Salmonella* is reduced if *sirA* is not functional; unlike other transcriptional regulators of TTSS-1, SirA is located outside the SPI-1 island (Johnston, Pegues et al. 1996).

The interaction of the effector proteins of TTSS-1 results in completion of the first stage of invasion of the host's epithelial barrier required for progression of the infection to the systemic phase.

1.2.2.3 Intracellular Survival and Replicative phase of infection

Upon internalisation into host cells, *Salmonella* is surrounded by a membrane-bound vacuole (Steele-Mortimer, Meresse et al. 1999). Recruitment of lysosome associated membrane proteins (LAMP1 and LAMP2) to replace the early endocytic markers follows, and *Salmonella* induced filaments (Sifs) also form (Garcia-del Portillo, Zwick et al. 1993; Garcia-del Portillo and Finlay 1994; Roark and Haldar 2008).Over the course of the next hour following entry of the bacteria, the vacuolar environment acidifies (Rathman, Sjaastad et al. 1996; Holden 2002).*Salmonella* controls the development of this compartment, then called the *Salmonella*-containing vacuole (SCV) (Haraga, Ohlson *et al*. 2008).

The SCV provides a relatively benign environment within which *Salmonella* is able to replicate. *Salmonella* Pathogenicity Island 2 (SPI-2),like SPI-1, was horizontally acquired during the evolution of the organism and encodes a second TTSS (Shea, Hensel *et al*. 1996), the effector proteins of which are critical for SCV formation and maintenance (Chakravortty, Hansen-Wester *et al*. 2002). It is interesting, therefore, that a major *Salmonella* variant, *Salmonella bongori*, lacks the SPI-2 apparatus. This suggests that during evolution of the different strains, *Salmonella enterica* and *Salmonella bongori* or their ancestors, split prior to the acquisition by *Salmonella enterica* of SPI-2 (Ochman and Groisman 1996). It has also been reported that another serovar, *Salmonella* Arizona, shows variability in the presence of SPI-2 which suggests that this strain also diverged separately from the other serovars (Chan, Baker et al. 2003). *Salmonella* replicates inside the SCV, ultimately leading to host cell death; the ensuing host cell lysis, accompanied by the release from the lysed cell of viable bacteria, may facilitate infection of neighbouring cells (Fink and Cookson 2007).Recent work using macrophage cells has demonstrated that when the single *Salmonella* within the SCV replicates, the SCV also divides and

replicates so that there is only ever one bacterial cell within a SCV, and the number of vacuolar compartments within the host cell increases (Lahiri, Eswarappa et al. 2010). None of the SCVs will fuse with lysosomes within the host cell and so avoid destruction. *Salmonella* is not alone in its ability to induce host cell death, and *Shigella* and *Yersinia* spp. also exhibit this capacity (Navarre and Zychlinsky 2000). The death and destruction of host cells by *Salmonella* itself then generates the release of inflammatory cytokines (Zychlinsky and Sansonetti 1997). SipB has been shown to be necessary for the induction of apoptosis by *Salmonella* (Hersh, Monack *et al*. 1999), and apoptosis results through the interaction of SipB with caspase-1, which is then activated. Because caspase-1 activates pro-inflammatory cytokines Interleukins 1-beta (IL1β) and 18 (IL18) (Sansonetti, Phalipon et al. 2000), the apoptotic cascade which follows results in the programmed cell death of infected macrophage cells. *Salmonella* Typhimurium, together with several other Gram-negative pathogens like *Shigella flexneri* and *Pseudomonas aeruginosa*, induces a specific type of cell death known as 'pyroptosis', from the Greek meaning 'pyro' – fire or fever, and 'ptosis' – falling, elegantly describing the inherent inflammatory characteristics of caspase-1-dependent cell death (Cookson and Brennan 2001). Such pyroptosis is also dependent on the SPI-1 TTSS, and the presence of bacterial flagellin (Fink and Cookson 2007). Strains which have mutations in either SPI-1 genes which encode TTSS (Monack, Raupach *et al*. 1996; Lundberg, Vinatzer *et al*. 1999; van der Velden, Lindgren *et al*. 2000)or which are non-flagellate (Franchi, Amer *et al*. 2006; Miao, Alpuche-Aranda et al. 2006), are not cytotoxic. Interestingly, Chen *et al* have also shown that translocated effector proteins sipA and sptP have no involvement in this pyroptotic pathway (Chen, Kaniga *et al*. 1996), since strains carrying mutations in these proteins are still able to cause pyroptosis. Pyroptosis is distinct from other types of apoptosis, which require the activation

of caspase-3, -6 or -8 (Brennan and Cookson 2000; Jesenberger, Procyk *et al*. 2000). It has also been shown that pyroptosis of *Salmonella* infected macrophages is not accompanied by the mitochondrial release of cytochrome *c* normally associated with apoptotic caspase activation (Jesenberger, Procyk *et al*. 2000).

The release of the caspase-1 dependent cleavage and activation of the IL1β and IL18 cytokines (Sansonetti, Phalipon *et al*. 2000; Monack, Detweiler *et al*. 2001) leads to inflammation associated activation of the host immune response. Such a full-blown response causes an influx of immune cells to the affected area and enhances T-cell and natural killer (NK) cell involvement (part of the innate immune response) (Braddock, Quinn *et al*. 2004). While neither IL1β nor IL18 are required to kill macrophages (Monack, Detweiler *et al*. 2001), they are instrumental in the production of the fever which accompanies the infection observed in the host, as described earlier (Section 1.1.5). The rapid cell lysis which occurs as a result of pyroptosis also provokes further increase of the inflammatory response, which can ultimately lead to the tissue destruction observed *in vivo*, manifested by intestinal haemorrhage and/or perforation (Everest, Wain *et al*. 2001; Hosoglu, Aldemir *et al*. 2004; Hosoglu, Celen *et al*. 2006) and abscesses (Gremillon, Geckler *et al*. 1977).

Salmonella is also able to survive and replicate within professional phagocytes, such as macrophages. A local influx of neutrophils, dendritic cells and macrophages is generated by the cytokine signalling by the innate immune system, in response to the presence of the bacterial stimulus (Tukel, Raffatellu et al. 2006).Neutrophils, or leukocytes, are the most common white blood cell, and the first to arrive at the site of an infection in response to chemotactic signals provided by cytokines, such as IL1β and IL18 (Levy, Koeppen et al. 2005).Their role is to ingest and destroy foreign particles and pathogens, and in doing so, they are themselves destroyed (Kindt 2007).Dendritic cells, (DCs) also

called antigen presenting cells (APCs) are, like neutrophils, phagocytic, but their main role is to isolate and process foreign antigens to present them to other cells of the immune system to generate a specific immune response (Levy, Koeppen et al. 2005). This is vital to the memory response engendered by immunisation. Macrophages are large phagocytic cells which ingest foreign particles and microbes, and form part of the reticuloendothelial system; they are stimulated by the presence of inflammation (Kindt 2007).

Macrophages normally phagocytose and kill invasive pathogens with strong oxidative and nitrosative bursts, which bombard the pathogen with lethal amounts of reactive oxygen intermediates (ROIs), nitric oxide and reactive nitrogen intermediates (RNIs), including nitrite and nitrous acid (Fang 1997). The production of NO which is mediated by the inducible Nitric Oxide Synthase (iNOS) in macrophages is an important protection against *S*. Typhimurium infection (Umezawa, Akaike et al. 1997). Mice lacking functional alleles of *iNOS* (*iNOS*$^{-/-}$) are significantly more susceptible to *Salmonella* infection relative to control mice (Mastroeni, Vazquez-Torres et al. 2000). During the course of infection of iNOS-deficient mice bacterial population growth in murine tissues is controlled at levels similar to those observed in wild type mice during the first week post infection. However, this is subsequently followed by lethal overgrowth of *S*. Typhimurium in the organs. Such a relatively "late" role for NO in the infection process is distinct from the "early" role of superoxide produced phagocyte oxidase (*phox*). Increased proliferation of *S*. Typhimurium in *phox*$^{-/-}$ mice is observed as early as 24 hours post infection (Mastroeni, Vazquez-Torres et al. 2000). *Salmonella* has evolved several mechanisms to overcome killing by NO (Mastroeni, Vazquez-Torres et al. 2000; Vazquez-Torres, Jones-Carson et al. 2000), which are discussed later. *Salmonella* which resist phagocyte killing are disseminated around the body within macrophages via the lymphatic circulation system. In systemic infections, *Salmonella* is spread via the reticulo-

endothelial system to the spleen, a secondary lymphoid organ, and to Kupffer cells dispersed throughout the liver (Nnalue, Shnyra et al. 1992). Kupffer cells are specialised liver macrophages (Kindt 2007). *Salmonella* bacteria ultimately induce pyroptosis of infected macrophages (Hernandez, Pypaert et al. 2003; Fink and Cookson 2007). The combination of both resistance to, and killing of, host cells, ensures the survival and dissemination of *Salmonella* during systemic infections (Detweiler, Cunanan et al. 2001). Figure 2 illustrates processes which occur during dissemination of *Salmonella* through the host immune system.

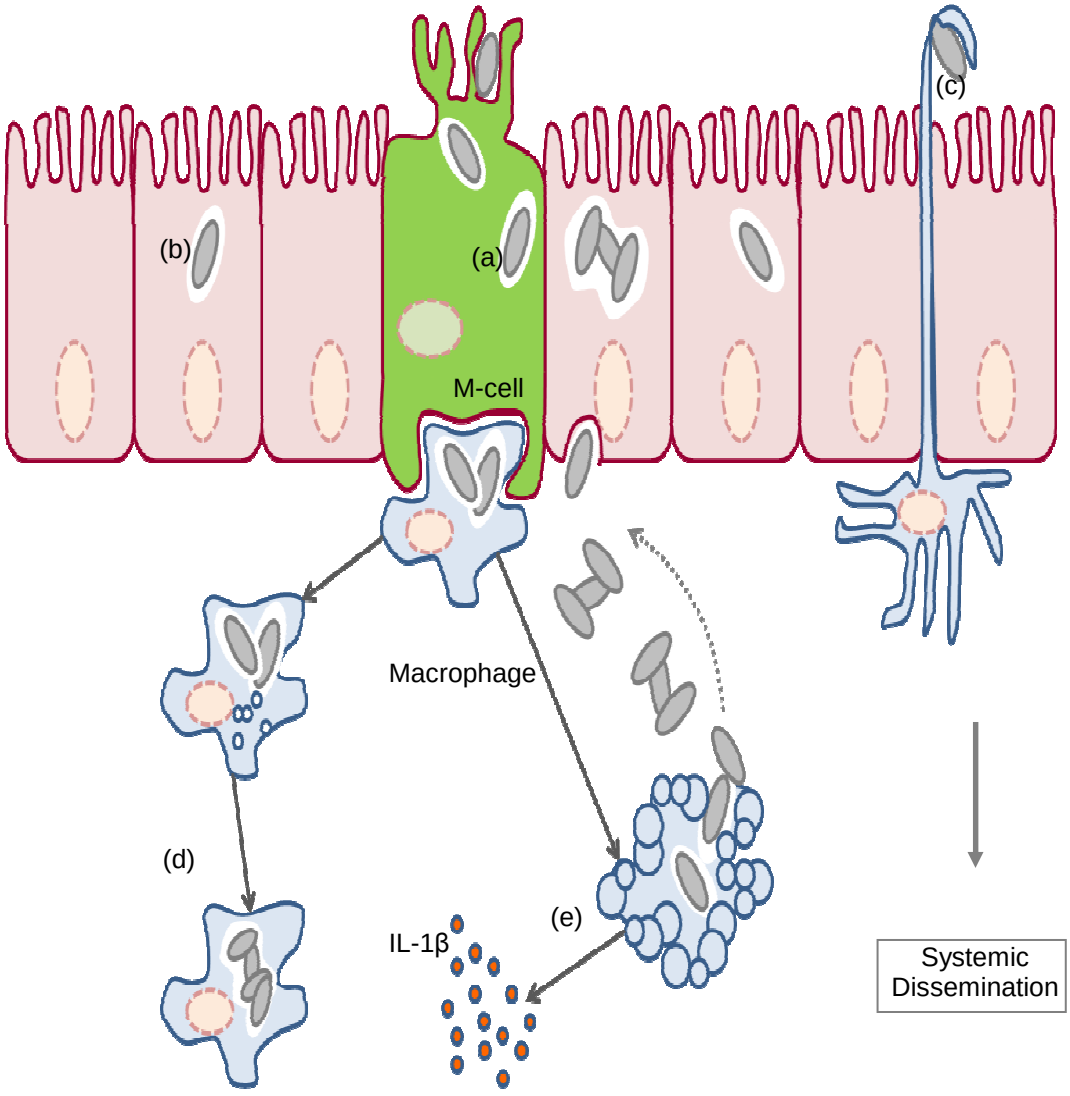


Figure 2 – Processes which assist the dissemination of *Salmonella* through the host system during the infection process. (a)The M-cell facilitates adhesion and translocation of *Salmonella* to the serosal side of the intestinal epithelium as part of its normal sampling function. In addition, *Salmonella* subverts the cytoskeletal structure of epithelial cells to gain entry directly (b), by employing its SPI-1 TTSS. Dendritic cells extend processes between the tight junctions sealing the epithelial barrier to sample gut contents; they may draw bacterial samples through to the sub-epithelium thus giving *Salmonella* an opportunity for systemic dissemination (c). Once inside the macrophage, *Salmonella* switches on SPI-2 effectors and assimilates cellular vesicles, which facilitate the construction of the modified Salmonella containing vacuole, within which it survives, grows and replicates (d).*Salmonella* might ultimately cause macrophage apoptosis which allows escape of viable bacteria which invade epithelial cells basolaterally, through use of SPI-1 machinery. Apoptosis of macrophages also triggers production of IL-1β, interleukin-1β, triggering the inflammatory response (e), and causing the recognisable intestinal symptoms associated with salmonellosis infection.(Adapted from (Sansonetti 2004))

1.3 Treatment and prophylaxis of *Salmonella* infections

Infection in humans by *S.* Typhimurium is generally self-limiting, with the gastroenteritic infection which it causes lasting between 4 and 7 days. In previously healthy patients, antibiotic treatment does not shorten this period. However, in the elderly or the very young, a systemic infection may ensue which does require intervention with antibiotics. This is further complicated where patients are immunocompromised, for example having undergone organ transplants or infected with the Human Immunodeficiency Virus (HIV). Some individuals may apparently recover from their symptoms, but then become chronic carriers of the infection, which they may periodically become infective for and pass on the infection to others, particularly if they are involved in food preparation. This was true of Mary Mallon, who during the early 1900s became known as 'Typhoid Mary'. She worked as a cook, and was responsible for the infection of at least 53 people with Typhoid Fever, of which three died. She was herself completely asymptomatic.

Current regimens for treatment of enteritis or food poisoning conditions, including salmonellosis are varied and can be controversial. Since the disease in an otherwise healthy individual is generally self-limiting, many doctors advocate letting the infection run its course, without any medical intervention using antibiotics. Other practitioners prefer to prescribe a course of antibiotics like ciprofloxacin for between 10 and 14 days. One problem with this approach is that it may prolong and/or exacerbate the carrier state. Antibiotic treatment, is however, generally agreed upon in those circumstances where the patient is immuno-suppressed for any reason. Pregnancy may also predispose an expectant mother to enteric infections. Certainly, if a fever or dehydration is suffered, they should expect extra support from their doctor, which may simply entail the administration of solutions to rebalance the electrolytic abnormalities

which may arise during prolonged bouts of diarrhoeal infections. In severe cases, administration of such medications may be favoured by the intravenous route. As described above in the case of Typhoid Mary, carriers can infect other people. Around 85% of carriers can be completely cured of their carrier status by surgical removal of their gall bladder, where the quiescent populations of bacteria reside, combined with antibiotic treatment.

1.3.1 Antibiotic Resistance

Of additional concern is the emergence and increase in numbers of multi-drug resistant variants of *Salmonella*, such as was identified as the infecting organism in the United States outbreak of December 2007. The phenomenon of antibiotic resistance has been a problem noticed early on following the introduction of antibiotics in the 1940s (JAMA 1960). Multi drug resistance is now becoming a worrying feature with many pathogenic bacteria, including *Salmonella* (WHO 2002). Other pathogens of major importance in terms of their resistance to antibiotic drug therapies are *Mycobacterium tuberculosis*(multi-drug resistant TB, or MDR-tb) (Telenti, Imboden et al. 1993; Murray 1994; Andersson and Levin 1999), the increasingly common nosocomial infection *Staphylococcus aureus*(particularly methicillin resistant *S. aureus*, MRSA) (Locksley, Cohen et al. 1982; Herold, Immergluck et al. 1998; Enright, Robinson et al. 2002; Okuma, Iwakawa et al. 2002), and the sexually transmitted infection caused by *Neisseria gonorrhoea* (Hook, Brady et al. 1989; Handsfield and Whitlington 1996).It is a natural part of the evolutionary process that where an organism meets a challenge, if one organism possesses traits which enable it to meet and overcome that challenge, then it will survive and is likely to pass its genetic advantage on to the next generation (Baquero 1997; Levin, Perrot et al. 2000; Blazquez, Oliver et al. 2002). In bacteria, which are able to reproduce

extremely rapidly, this ability can become noticeable fairly quickly after the challenge has been met, and is known as vertical evolution (Saunders 1984) which arises by the growth in the population of the bacteria. A major influence has been the free prescribing of antibiotics when their use is relatively unnecessary, such as might happen in a *Salmonella* infection of an otherwise healthy individual (Ridley, Lynn et al. 1970; Salyers and Amabile-Cuevas 1997). It is also common for patients to fail to complete the prescribed course of antibiotics, which increases the chances of survival of viable, resistant bacteria (Magee, Pritchard et al. 1999; Goossens, Ferech et al. 2005).This arises because less sensitive bacteria have the opportunity to multiply and eventually emerge as fully resistant strains. Antibiotic therapies are designed to compromise vital processes which normally occur to ensure survival of bacteria. The targets of the antibiotic may be the bacterial pathways involved in transcription, protein or cell wall synthesis or other metabolic processes. Penicillin, for example, affects the ability of microbes to synthesise cell wall components (Wise and Park 1965; Tomasz and Waks 1975). Antibiotic resistance is also acquired by the transfer of resistance (R) plasmids, which occurs during conjugation between bacteria, and is not dependent on growth of the bacterial population. This transfer of genetic material between bacteria is known as horizontal evolution (Beaber, Hochhut et al. 2004; Sorensen, Bailey et al. 2005), and can confer resistance to several antibiotics in one plasmid. A third way in which antibiotic resistance can be transferred is by the acquisition of a 'pathogenicity island', a large genetic element flanked on either side by transposable elements and containing genes for the expression of a range of factors which contribute to virulence and successful infection (Ochman, Soncini et al. 1996). It is thought that horizontal evolution has played a major role in the acquisition by *Salmonella* spp., as well as other species, of pathogenicity islands

(Ochman and Groisman 1996; Hacker, Blum-Oehler et al. 1997; Hensel 2000), with spontaneous mutations potentially playing an additional minor role.

1.3.2 Resistance to antibiotics in *Salmonella*

Since the 1960s, when antibiotic resistance was identified in *Salmonella* (Schroeder, Terry et al. 1968; Bulling, Stephan et al. 1973; Sojka and Hudson 1976), multidrug resistant strains have also emerged, and are becoming an increasing problem (Anderson 1968; Threlfall, Ward et al. 1997; Velge, Cloeckaert et al. 2005). It is thought that one of the major reasons for the increase in the resistance to antibiotic intervention in *S*. Typhimurium is the overuse of antibiotics in the livestock, poultry and fish farming industries (Davis, Hancock et al. 1999; Velge, Cloeckaert et al. 2005). Interestingly, in the case of *S*. Typhi and Paratyphi, it is more likely that the use and/or misuse of antibiotic therapies in human treatments has resulted in the increases in antimicrobial resistance seen in these pathogens (Goldstein, Chumpitaz et al. 1986; Parry, Wain et al. 1998; Threlfall 2002; Roumagnac, Weill et al. 2006). More recent work has also shown a correlation between different levels of metal contaminations, of various metals, and antibiotic resistance (Baker-Austin, Wright et al. 2006), which is also linked to the human pathogenic strain *S*. Typhi CT18. Indeed, metal contamination may serve as a selective agent in the spread of antibiotic resistance. Baker-Austin *et al* have postulated that this could be due to either the presence of different resistance functions on the same genetic element (co-resistance) or the same genetic determinant having a function which confers resistance to both antibiotics and metals (cross-resistance).

This shows another selection pressure which can contribute to the development of further antimicrobial resistance in *Salmonella*. Add to these features the

simple fact that *Salmonella* has a tough metabolism which has evolved over the millennia to survive a wide range of challenges (Becker, Selbach et al. 2006) and it is not surprising that the need for continual detection, identification and development of new molecular targets remains.

Since 1990, there have been laboratory reports of the emergence of a multidrug resistant strain of *Salmonella* Typhimurium definitive type (DT) 104 (Wall, Morgan et al. 1994). These have been associated with many and varied zoonotic reservoirs from cats (Wall, Threllfall et al. 1996) to cattle (Low, Hopkins et al. 1996; Hollinger, Wray et al. 1998; Davis, Hancock et al. 1999), seals (Foster, Ross et al. 1998) to pigs (Baggesen and Aarestrup 1998; Rugbjerg, Wingstrand et al. 2004), and chickens (Rajashekara, Haverly et al. 2000) to, quite recently, rabbits (Borrelli, Fioretti et al. 2010). Isolates of phage type DT104 are often resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and also tetracycline, described as ACSSuT resistance. Concern also arises around the ability of this strain to resist eradication by antibiotics as a result of the ability of the Salmonellae to both gain and donate horizontally transferred regions of their genetic makeup (Briggs and Fratamico 1999; Beaber, Hochhut et al. 2004; Sorensen, Bailey et al. 2005). This mechanism is believed to have operated during the acquisition by *Salmonella* of the pathogenicity islands associated with invasion (Ochman and Groisman 1996; Hensel 2000). Although this strain may have developed wide antibiotic resistance through extensive use of antibiotic regimens in the livestock industry, if it subsequently causes infection among exposed human populations it carries the resistance with it and consequently such human infections are increasingly difficult to treat successfully. The antibiotic resistance observed in DT104 has also been shown to have extended to several other serovars of *Salmonella enterica* which carry a class 1 integron within genomic island SGI1, or variants of it containing different combinations of resistance genes (Levings, Lightfoot et

al. 2005), and all of which have been isolated from human infections. The DT104 variant is also more virulent than other strains, which adds to the problem, since the severity of any infection caused by DT104 is also increased. It is also possible that horizontal transfer will occur between species of bacteria, thus spreading the antibiotic resistance capability still further, and the likelihood that such a multi resistance gene fragment could be transferred to another bacterial pathogen should cause concern (Briggs and Fratamico 1999).

In consequence of the various factors which are contributing to the development of resistance to antibiotic regimens, we are faced with increased difficulty in ensuring effective treatment of infections caused by *Salmonella* species, In some less economically developed countries, *Salmonella* infections are endemic. In addition, as global travel becomes more common and accessible, more opportunities for widespread dissemination of *Salmonella*-associated infection can be envisaged. As a result, there is an urgent need to identify new bacterial targets to enhance the development of novel antimicrobial therapies.

1.3.3 Vaccines

While it is possible to use *Salmonella* vaccines to immunise against Typhoid infections, they also have potential to be used as vaccine vectors or multivalent vaccines to deliver foreign antigens. The strains which are used for vaccines need to be stable, and provide long term protection, but there is a difficult balance between their ability to provoke an immune response (immunogenicity) and their capacity to produce an adverse reaction (reactogenicity) which must be achieved (Everest, Griffiths et al. 1995; Pasetti, Levine et al. 2003).The World Health Organization commissioned several trials during the 1960s of two potential vaccine candidates in areas where typhoid was endemic at that time, in Poland, the USSR, Yugoslavia and British Guyana (Cvjetanovic and Uemura

1965). This compared the performance of a heat-phenol inactivated vaccine to an acetone-dried inactivated vaccine. Both gave good protection in the short term, but results were not so notable in a long term study carried out later in Tonga (Tapa and Cvjetanovic 1975), and both vaccines caused side effects which ranged from injection site reactions to headache and fever over 1 to 2 days. Such reactions tended not to prevent return of the participants for the follow-up injection. Other work also considered the differences in efficacy between heat-killed, chemical and alcoholised vaccines (Hejfec 1965), and found that while the heat-killed vaccine was more protective than either of the other two, that difference was probably mostly dose dependent, as were the reactogenicity levels. Ultimately, this reactogenicity has been linked to the bacterial LPS which remains in these 'whole cell' preparations (Hoops, Prather et al. 1976).

The World Health Organisation advocates the use of two typhoid vaccines which are currently licensed worldwide; one, based on the Vi polysaccharide of *S.* Typhi is given parenterally, and the other, Ty21a is a live vaccine using an attenuated strain of *S.* Typhi which is administered orally (WHO 2009). Only the parenteral vaccine is approved by NICE for use in the United Kingdom.

An interesting adjunct is that as a result of intensive annual vaccination of children in Asia, which use the Vi vaccine, a dramatic reduction in the number of cases of typhoid has been observed, across age groups which have not been specifically targeted by the vaccination programmes. This suggests that herd immunity is arising as a result of effective immunisation programmes (WHO 2009).

With the ongoing development of resistance to antibiotic treatment which is evident, there is no room for complacency, and the importance of making continued advances in the area of prevention of *Salmonella* infections should not be underestimated (DeRoeck, Jodar et al. 2007; Chakravortty 2010).

1.3.3.1 Purified Vi Polysaccharide

The potential of the Vi capsule of *S.* Typhi as a protective antigen was investigated, and it was shown that earlier failed attempts to produce a vaccine using the Vi capsule had processed the Vi antigen in such a way that the protein was denatured (Robbins and Robbins 1984), and consequently incapable of initiating the desired immunogenic response. They developed a non-denaturing method for large scale purification of the capsule, which resulted in the development of a vaccine which has been in use since 1990. The vaccine has been shown to be highly effective, and suitable for use in young children, since it is much less reactogenic than it's whole-killed-cell predecessor (Acharya, Lowe et al. 1987; Klugman, Gilbertson et al. 1987; Klugman, Koornhof et al. 1996).Because of the requirement for a booster dose to be given every three years with this vaccine, it is not ideal in many regions of the World where Typhoid Fever is endemic. Consequently, work has continued and ongoing investigations continue to explore and maximise the efficacy of this type of vaccine (House, Ho et al. 2008; Kothari 2010; Staats, Kirwan et al. 2010)

1.3.3.2 Live Ty21a

A live, but attenuated, strain of *S*.Typhi provided the first oral vaccine used to combat Typhoid Fever (Germanier and Furer 1975).This work initially attributed the avirulence of the strain, called *S*. TyphiTy21a, to the loss of functional *galE*, which resulted in the erroneous uptake of galactose which ultimately resulted in the lysis of the cells. However, it has subsequently been suggested that there are probably multiple mutations in the strain, due to the chemical method used to induce the attenuated phenotype (Silva, Gonzalez et al. 1987). Indeed, in addition to Vi capsular mutations and nutritional auxotrophies (Cryz and Furer 1988), Ty21a is also deficient in *rpoS* (Robbe-Saule, Coynault et al. 1995). A

strain mutant only in *galE* also retains its virulence in humans (Hone, Attridge et al. 1988).

The Ty21a vaccine requires several doses to generate immunity (Levine, Ferreccio et al. 1987), confirmed in work which also showed that the material used to make the capsule to contain this oral vaccine also affects its efficacy. However, it concluded that the Ty21a oral vaccine was a preferable tool to the whole killed cell alternative due to the lower reactivity which it caused in subjects.

Because of its modest immunogenicity, and the fact that even after multiple doses, not all patients are protected from Typhoid Fever (Simanjuntak, Paleologo et al. 1991), it is necessary for the search for alternative and improved prophylactic solutions to be continued.

1.3.3.3 Other Live Oral Vaccine Candidates

In those regions of the World where Typhoid Fever presents the greatest risk to health, it is often also the most difficult to transport, store and administer vaccines safely. Often, other infections, such as HIV may also be prevalent in these areas, so the least invasive routes through which to provide immunisation are preferable. Consequently, oral administration is to be favoured, where efficacious candidates can be identified. Ideally, *Salmonella* strains can be modified so that while they are incapable of causing an infection, they are immunogenic enough to induce a protective immune response after only a single dose is administered. Staats *et al*. showed that oral dosing engendered both serum IgG and a secretory IgA response (Staats, Jackson et al. 1994). The gut mucosal system is critical to the body's defence against, in particular, pathogens which cause enteric infections and diarrhoeal disease. In 1981, Hoiseth and Stocker proposed that a *S*. Typhimurium strain defective in *aroA*

and avirulent in mice, would be a good candidate for use as a live oral vaccine (Hoiseth and Stocker 1981). Later, the same aromatic pathway was mutated in *S*. Typhi by the deletion of *aroA* and *aroC*, to produce strain WBL2000 (Dougan, Chatfield et al. 1988), with later work refining the mutations further to produce vaccine strains CVD906 and CVD908, lacking *aroC* and *aroD* (Hone, Harris et al. 1991). Further work characterized strains BRD691 (mutated in *aroA* and *aroC*) and BRD1116 (with mutations in *aroA*, *aroC* and *htrA*), and showed the potential for use of these strains both as typhoid vaccines and vaccine vectors for other or multiple pathogens (Tacket, Sztein et al. 1997; Lowe, Savidge et al. 1999). Later work has further engineered the strains to limit the virulence of potential vaccine strains by mutating the SPI-2 pathways, for example by deletion of *aroC* and *ssaV* (strain *S*. Typhi Ty2 ZH9), to provide highly attenuated yet immunogenic candidates for both human typhoid vaccines and potentially for delivery of heterologous antigens as vaccine vectors (Khan, Stratford et al. 2003).

1.3.3.4 Vaccines for Other Diseases

Along with other attenuated bacterial variants, *Salmonella spp.* have been investigated extensively as likely vectors (Curtiss, Galan et al. 1990; Galen, Nair et al. 1999; Stephens, Darsley et al. 2006). The application of recombinant DNA techniques makes it possible for engineered strains of *S.* Typhi to express a range of antigens from other species which cause human disease, and hence to be exploited as oral vaccine vectors for afflictions other than Typhoid Fever. The fact that *S.* Typhi provides an efficient vehicle for oral immunisation makes them safer and easier to administer, and any risks or complications which arise due to the use of parenteral methods in connection with other blood-borne pathogens, such as HIV in many parts of the World, are minimised. Also, as mentioned

previously (Section 1.3.3.3), *Salmonella* vectors also induce powerful humoral and cellular responses from the immune system, helping to ensure effective immunogenicity (Levine, Galen et al. 1996). The possibility that such vaccines can also be used to immunize populations against several diseases in one dose, is also highly attractive both from an economic and practical standpoint (Tacket, Sztein et al. 1997; Roberts, Li et al. 1998). Recent work, in mice using an attenuated strain of *S*. Typhimurium, also suggests that an alternative mucosal route to oral administration may be by intranasal dosing which could be accomplished in a fairly simple spray (DiGiandomenico, Rao et al. 2007). Progress is also being made with vaccines to offer protection against Human Immunodeficiency Virus (HIV) infections using *Salmonella* or *Shigella* vectors (Devico, Fouts et al. 2002; Chin'ombe, Bourn et al. 2009).

Another exciting possibility is that of using *Salmonella* vectors to deliver anti-tumour therapeutics directly to the site, or sites in metastatic disease, of cancerous, and possibly malignant, tumours (Pawelek, Low et al. 1997); potentially, *Salmonella* as a RNA interference vector (Basu and Herlyn 2008) could also be employed to deliver cancer gene therapies.

Recent observations suggest that some disappointing results in human trials of some of these vector vaccines may, at least in part, be due to the over-attenuation of vaccine vector strains which has caused a loss of immunogenicity (Galen, Pasetti et al. 2009). However, with continued improvements in techniques, and the identification of more novel variants in which stability of the expression of antigens can be balanced with effective immunogenicity, further advances in this field will be possible.

1.4 *Salmonella* metabolism

Salmonella is able to grow under both aerobic and anaerobic conditions (Yamamoto and Droffner 1985; Aliabadi, Warren et al. 1986; Gunsalus and Park 1994). It is facultatively anaerobic, which means that it can carry out all necessary metabolism without oxygen, although when oxygen is available, it prefers to use that as the terminal respiratory chain electron acceptor until it has been exhausted, when it will switch to anaerobic respiration. Unlike strict anaerobes, such as *Clostridium perfringens* (Stickland 1934; Marrie, Haldane et al. 1981), or microaerophiles like *Campylobacter jejuni* (Lander and Gill 1980) or the closely related pathogen *Helicobacter pylori* (Pesci, Cottle *et al*. 1994*),* the presence of oxygen at increasing concentrations is not lethal to *Salmonella*. Its ability to switch to anaerobic metabolic activity means that *Salmonella* can then use other electron acceptors, such as nitrate. Naturally, this ability confers an advantage to *Salmonella* since as an enteric pathogen, it encounters a wide range of oxygen concentrations during its passage through the gastrointestinal tract. Until recently, this internal environment was thought to be largely anaerobic, but current thinking is that there are also many microaerobic niches within the gut (Jones, Chowdhury et al. 2007).

Interesting work has recently reported that while a complete TCA cycle is advantageous to *Salmonella* during its occupation of epithelial cells, it survives better in macrophages if the TCA cycle has been interrupted (Bowden, Ramachandran et al. 2010). Bowden *et al* postulate that this may be linked to the inability of epithelial cells to mount a response through reactive oxygen or nitrogen intermediates, while that is a feature of the macrophage response to infection. They conclude however, as a result of the attenuation of such mutants in mouse infection, that a complete TCA cycle is necessary to ensure survival in

a range of different environments which may be encountered in the course of an infection.

1.4.1 Metabolism of Glucose by *Salmonella*

The favoured carbon source for most microorganisms is glucose, which contains six carbon molecules. The presence of glucose, even with another useable carbon source, often results in repression of the pathways capable of using the second source, until the glucose supply has been exhausted (Magasanik 1961). In *Salmonella*, (though also in *E. coli*), glucose is transported via the phosphoenolpyruvate : phosphotransferase system (Kaback 1968; Postma, Neyssel et al. 1982).Aerobic glucose metabolism produces 38 Adenosine Trisphosphate molecules (ATPs), the energy currency of the cell, from each glucose molecule; in contrast, only two ATPs are released under anaerobic conditions. This is because in the absence of oxygen as an electron acceptor, not all the carbon molecules can be oxidised to carbon dioxide. *Salmonella* makes use of the glycolysis fermentation pathway to release energy from glucose, which is less efficient than the aerobic pathway.

Critically, *Salmonella* must also be able to metabolise successfully within the macrophage environment, and Bowden *et al* have shown that *S*. Typhimurium requires glycolytic processes and the transport of a supply of glucose within the SCV of macrophages (Bowden, Rowley et al. 2009). This was demonstrated by the inability of mutants in which vital genes involved in sugar transport and catabolism had been deleted to successfully replicate in macrophages or infect mice.

1.4.2 Metabolism of Glycerol by *Salmonella*

In contrast to glucose, glycerol consists of a three-carbon chain, and unusually for carbohydrate transport, requires facilitated diffusion via a pore channel to enter *E. coli* (Heller, Lin *et al*. 1980). Anaerobic glycerol metabolism also frees two ATP per molecule of glycerol.

1.4.3 Nitrate Metabolism by *Salmonella*

Salmonella is able, along with *E. coli*, to use nitrate as a terminal electron acceptor during anaerobic respiration (Stewart 1988; Stewart 1993; Glaser, Danchin et al. 1995). Other bacteria, for example *Pseudomonas aeruginosa*, and including the enteric bacterium *Klebsiella pneumonia* also assimilate nitrate (Campbell and Kinghorn 1990). During assimilation of nitrate, reduction through nitrite to ammonium can provide the sole nitrogen source. Nitrate assimilation and anaerobic nitrate respiration are the two forms of nitrate reduction which occur in microbial metabolism.

1.4.3.1 Nitrate Assimilation

Nitrate is an important source of nitrogen for many microorganisms, including *Salmonella* (Lin and Stewart 1998). To enable microorganisms to handle nitrates safely, they possess a range of enzymes which are expressed under conditions where nitrate is present. Nitrate reductases comprise iron-sulphur clusters and a molybdenum cofactor, while nitrite reductases, which facilitate the next stage of nitrate reduction and detoxification incorporate sirohaem with iron-sulphur clusters (Lin and Stewart 1998). However, during aerobic growth, neither *Salmonella*nor *E. coli* are able to assimilate nitrate (Ingledew and Poole 1984; Stewart 1994; Glaser, Danchin et al. 1995; Stenuit, Eyers et al. 2006).

1.4.3.2 Anaerobic Nitrate Respiration

A combination of two systems, anaerobic respiratory nitrate reduction and nitrite reduction, covered later, allow *Salmonella* and *E. coli* to use nitrate as a sole nitrogen source under anaerobic conditions (Neidhardt 1996).

1.5 *Salmonella* and Reactive Nitrogen Species (RNS)

The environment within the mammalian gastrointestinal tract has been shown to be largely anaerobic, and to contain high levels of nitrate. Since nitrate may act as a terminal electron acceptor for respiration in the absence of oxygen (Lundberg, Weitzberg et al. 2004), it could be anticipated that a link exists between nitrate reductase activity and *Salmonella* survival in the gut (Contreras, Toro et al. 1997). However, recent work using respiratory mutants of *E. coli* revealed that the intestinal environment varies between microaerobiosis and anaerobiosis (Jones, Chowdhury et al. 2007), since the cytochrome *bd* oxidase, which has a high affinity for oxygen, is vital for successful colonization. Consequently, it seems possible to deduce that the mammalian gut comprises both anaerobic and microaerobic niches. Interestingly, it has been shown that the terminal respiratory oxidase cytochrome *bd* in *E. coli* is potently inhibited by nitric oxide (NO) (Borisov, Forte et al. 2004).

1.5.1 Reactive Nitrogen Species (RNS): an important component of the innate immune system

1.5.1.1 Innate Mammalian Defence

Innate host defence systems respond non-specifically to the presence of pathogens. The responses do not confer long-lasting or protective immunity or the establishment of immunological memory, such as occurs in the adaptive immune response (Levy, Koeppen et al. 2005). However, activity of the innate

defences may later result in activation of the adaptive immune system, through presentation of antigens (Kindt 2007).

Innate immunity includes barrier defences, such as the integrity of the skin, which prevents entry of pathogens. The skin surface is also maintained at a slightly acidic pH by secretions, produced by hair follicles which contain lactic acid and fatty acids (Maggini, Wintergerst et al. 2007). Ciliary activity in the lungs expels foreign particles, including pathogenic microbes, by beating of the hair-like cilia in an upward direction (Levy, Koeppen et al. 2005). Coughing and sneezing responses also expel irritants. Mucus is produced in both the respiratory and gastrointestinal tracts, and this can trap microbes, preventing their further activity (Levy, Koeppen et al. 2005). The normal flushing by tears, saliva and urine also removes pathogens. Indeed, tears and saliva contain lysozyme which can destroy the cell membrane of Gram-positive bacteria, leading to bacterial lysis (Abergel, Monchois et al. 2007). The immune processes function together to help mammals to prevent infection by bacterial pathogens.

1.5.1.2 Stomach Acidity and RNS

One of the first innate mammalian defences to be encountered by ingested enteric pathogens is the acidic environment of the stomach. Here, acidity may dip as low as pH1 in the immediate post-prandial period (Levy, Koeppen et al. 2005; Rychlik and Barrow 2005). Bacterial survival of the transit through the stomach is achieved through activation of the acid tolerance response (ATR), the mechanisms of which have been discussed in more detail previously.

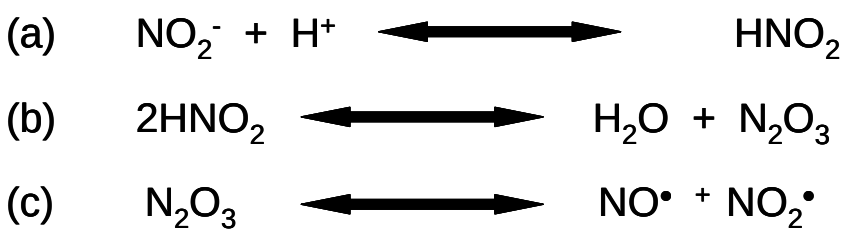
As well as directly stressing the bacteria, the acidity of the stomach converts dietary and salivary nitrite to NO, to produce nitrosative stress which the bacteria

must also survive. Nitrosative stress can cause changes to bacterial proteins which inhibits their normal functions, or inhibit DNA replication (Bang, Liu et al. 2006), rendering the bacteria non-viable. Exogenous nitrogen species are introduced to the gut in the diet. Most dietary nitrate present in the gastrointestinal tract is produced from vegetables (Lundberg, Weitzberg et al. 2004); beets, celery and leafy vegetables are especially rich in nitrates (Bryan 2006). In the oral cavity, salivary nitrate is reduced to nitrite by commensal bacteria on the tongue. In the stomach, the nitrite is acidified in a reaction with stomach acid, to nitrous acid, and subsequently by disproportionation to other nitrogen species, including NO (Equation 1) (Benjamin, O'Driscoll et al. 1994; van Wonderen, Burlat et al. 2008). Nitrite is also ingested in the diet, most often with cured and processed meats, to which nitrite is added as a preservative (Bryan 2006). Nitrite has also been shown to enhance mucus production at the epithelial surface of the gut (Bjorne, Petersson et al. 2004), which has ramifications for bacteria present in the gut which have already been described.

Residual nitrate and nitrite are ultimately excreted in the urine, at levels similar to those ingested in the diet (Lundberg, Weitzberg et al. 2004), ensuring that in the normal, uninfected system, a steady state of nitrate and nitrite levels is maintained. Faeces and sweat have been shown to constitute only minor routes for excretion of nitrate and nitrite ions (Weller, Pattullo et al. 1996; Ten Bruggencate, Bovee-Oudenhoven et al. 2004). Nitrogen species are consequently found distributed throughout the length of the gastrointestinal tract, representing a serious problem for enteric pathogens, and potentially also for commensal bacteria. However, the presence of the commensal bacterial community may itself also generate production of reactive nitrogen species by enterocytes of the mammalian mucosa, (Macpherson, Martinic et al. 2002). This means that as well as competing with pathogens for space in the intestine,

commensal bacteria ensure a background response from the host immune system is switched on.

Equation 1 – The disproportionation of Nitrite to Nitric Oxide



1.5.1.3 Macrophages and RNS

NO is produced by the normal constitutive activity of the L-arginine-NO pathway, which maintains various physiological functions like vascular tone, neurotransmission and platelet function (Levy, Koeppen et al. 2005). Endogenous NO is vital as a signalling molecule for many processes in the mammalian system, and is produced from the amino acid L-arginine and molecular oxygen by NO synthases (NOS) (Nelson and Cox 2004). There are three NOS isoforms; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Moncada, Higgs et al. 1997). Basal plasma NO levels are generated by the endogenous L-arginine-NO pathway. The NO can be detoxified by reacting with oxy-ferrous haemoglobin (Hb(FeII)) (Gow and Stamler 1998), the form of haemoglobin in which oxygen can bind to it, and in mammals, the NO reacts with oxidized haemoglobin in red blood cells, to produce nitrate (Lundberg, Weitzberg et al. 2004).

Macrophages use iNOS to produce NO, without the need for elevated intracellular Ca^{2+} which is required by eNOS and nNOS (Marletta 1994; Nathan

and Xie 1994; Griffith and Stuehr 1995; Michel and Feron 1997). Macrophages are mononuclear phagocytic leukocytes, which develop by differentiation from monocytes circulating in blood plasma. They operate in both innate and adaptive immunity. Some macrophages are migratory, flooding in to the site of an infection or to a breach in the immune barriers, while others are located in specific tissues, such as the Kupffer cells in the liver (Kindt 2007). Macrophages form a crucial part of the early response of the immune system to attack by pathogens, and production of NO is a vital part of the macrophage armoury. Macrophages produce copious amounts of NO from iNOS activation, in response to the presence of interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), which are cytokines generated at the initiation of an immune response (Busse and Mulsch 1990). Another cytokine, gamma-interferon (IFN-γ) stimulates NO production via IFN-γ receptor signalling. IFN-γ signalling causes the dimerization of Janus kinase (JAK) proteins, present in the macrophage cell membrane, and the subsequent recruitment of STAT proteins. Phosphorylation of the STAT proteins causes activation, dimerization and translocation to the cell nucleus, resulting in increased expression of the IRF-1 transcription factor. IRF-1 binds to the iNOS gene promoter region of the DNA and upregulates iNOS gene expression (Figure 3).

The main modulator of NO production is Transforming Growth Factor beta (TGF-β); since NO is also toxic to host cells, NO production must be tightly regulated (Berg, Gupta et al. 2007). The consequence of a systemic inflammatory reaction is the elevation of the basal level of endogenous production of NO as part of the defence mechanism, by the induction of iNOS in macrophages that have been challenged. Oxidation of NO by oxidized haemoglobin can increase the levels of nitrate in plasma; the nitrate is secreted in saliva, to be reduced in the oral cavity to nitrite as explained earlier. As shown in Equation 1, the nitrite will be disproportionated to produce NO. Consequently,

the levels of NO in the lumen of the gastrointestinal tract are also increased by the mammalian immune-mediated systemic inflammatory response. In patients with severe inflammation due to acute pancreatitis, total nitrite excretion is elevated (Rahman, Ammori et al. 2003), probably in response to the up-regulation of iNOS activity. Inflammation caused by *Salmonella* infection (Stecher and Hardt 2008) could also increase the amounts of NO to which remaining bacteria in the gut are exposed. Increased levels of NO may also be toxic to non-pathogenic bacteria in the gut, and alter the balance of the normal gut flora. Host-mediated inflammation, such as that which occurs during the course of an infection by *Salmonella*, also disrupts the normal intestinal microbiota, and consequently upsets the normal equilibrium which ensures homeostasis within the gut. This can result in the provision of a competitive advantage to pathogenic enterobacteria (Lupp, Robertson et al. 2007; Stecher, Robbiani et al. 2007; Henard 2011).

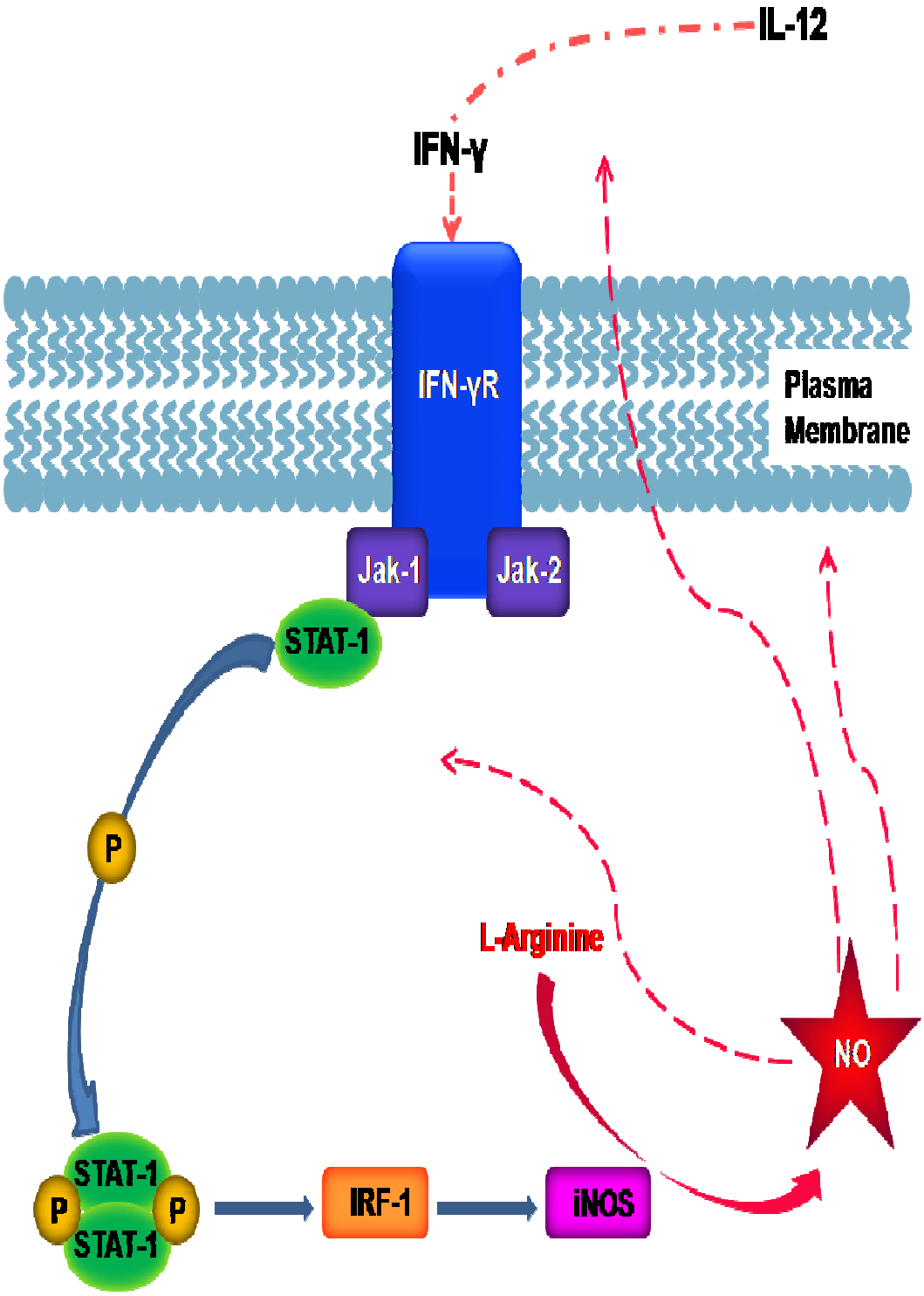
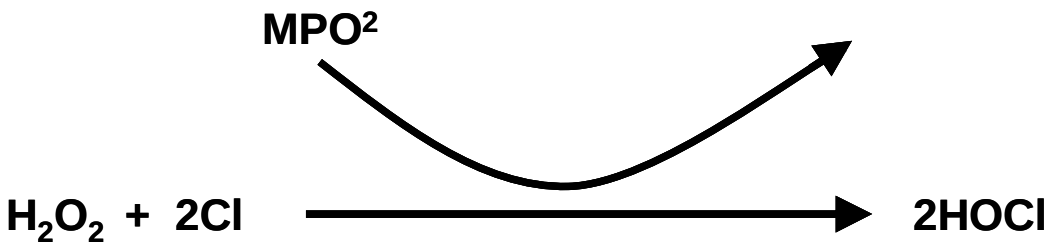


Figure 3 - **The signalling cascade for iNOS activation in macrophages.** Gamma-interferon (IFN-γ) stimulates NO production by dimerising IFN-γ receptor (IFN-γR) and Janus kinase (Jak) proteins, present at the macrophage cell membrane. STAT proteins are recruited and phosphorylated to activate them; they also dimerise and translocate to the cell nucleus, increasing expression of the IRF-1 transcription factor. IRF-1 binds to the iNOS gene promoter region of the DNA, upregulating iNOS gene expression, resulting in NO production. NO is able to diffuse through the macrophage plasma membrane

Macrophages also produce reactive oxygen species (ROS) to attack invading microbes, in addition to generating nitrosative stress. The NADPH oxidase enzyme bound in both the plasma and phagosome membranes of macrophages uses one electron of molecular oxygen to form superoxide, a highly reactive free radical. In phagosomes, superoxide can spontaneously convert to hydrogen peroxide, to produce other ROS. One possible product is hypochlorous acid, the effective agent in bleach which is used to kill bacteria (Roos and Winterbourn 2002; Winterbourn, Hampton et al. 2006). Hypochlorous acid production is catalysed by myeloperoxidase ($(MPO)^2$) acting upon chloride and hydrogen peroxide in the phagosome (Winterbourn, Hampton et al. 2006), as shown in Equation 2.

Equation 2 – The catalysis of hydrogen peroxide and chloride to hypochlorous acid



To summarize, the intraphagosomal environment of the macrophage is potentially lethal to most invading bacteria. However, *Salmonella* employs a range of defence mechanisms to avoid, and ultimately, exploit, the characteristics of macrophages. Primarily, *Salmonella* uses the SPI-2 TTSS, which allows the secretion of effector proteins to inhibit normal macrophage function (Chakravortty, Hansen-Wester et al. 2002).

1.6 Nitric Oxide (NO) Detoxification

The free radical NO• is highly reactive, and can form other toxic reaction intermediates and reaction products, or reactive nitrogen intermediates (RNIs) that enhance NO activity as an antimicrobial compound (Chakravortty and Hensel 2003). Mutations resulting from NO-induced damage to DNA have been observed in *S. enterica* (Wink, Kasprzak et al. 1991). In eukaryotic pathogens like *Leishmania major*, NO inhibits enzymes of the citric acid cycle (aconitase) (Green, Nacy et al. 1991). To overcome this stress, *Salmonella* employs at least three mechanisms, including the metalloenzymes cytochrome *c* nitrite reductase (*nrfA*), flavorubredoxin (*norV*) and flavohaemoglobin (*hmpA*). The activities of each of the three enzymes differ in their oxygen requirement. The anaerobic respiratory enzyme, NrfA, is only expressed and active in anoxic (an environment depleted of oxygen) or micro-oxic (an environment with low levels of oxygen present) conditions, and reduces either nitrite or NO to ammonium. NorV reduces NO to nitrous oxide (N_2O) in either anaerobic or low oxygen conditions. HmpA acts in oxic (replete in oxygen) environments to oxidize NO to nitrate (Gardner and Gardner 2002), or in anaerobic conditions it reduces NO to N_2O. In order to successfully manipulate mammals as hosts, pathogenic bacteria have evolved strategies for the detoxification of NO and the evasion of the ravages of the mammalian immune response. The resistance mechanisms used by *Salmonella* involve cytochrome *c* nitrite reductase, flavorubredoxin, and flavohaemoglobin and are described below. Figure 4 further illustrates the interplay between the various enzymes involved in NO detoxification.

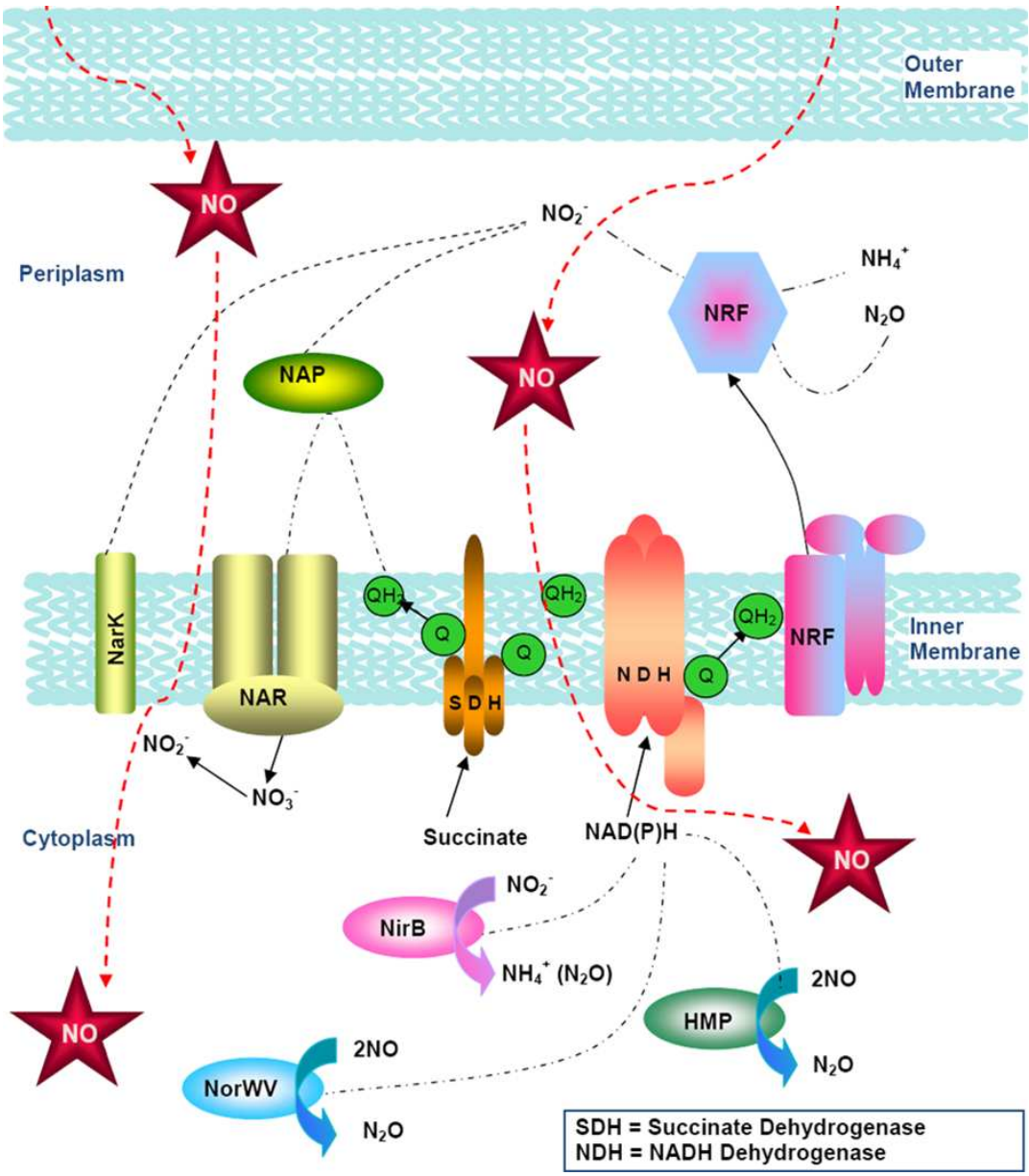


Figure 4 - Ammonification respiratory pathways in *Salmonella.*
NO produced exogenously is able to diffuse across both the outer and inner membrane of *Salmonella.* NO is detoxified anaerobically by enzymes such as cytochrome *c* nitrite reductase (*nrfA*), flavorubredoxin (*norV*) and flavohemoglobin (*hmpA*); *hmpA* may also work aerobically. The pathways of each enzyme differs in their oxygen requirement; NrfA is expressed in anoxic or micro-oxic conditions, and reduces either nitrite or NO to ammonium; NorV reduces NO to nitrous oxide (N_2O) in either anaerobic or low oxygen conditions; HmpA acts in oxic environments to oxidize NO to nitrate. In anaerobic conditions HmpA reduces NO to N_2O. NirB is the large subunit of the assimilatory nitrite reductase NAD(P)H, which associates with the small subunit, NirD. NirB is activated in anaerobic conditions and reduces NO_2^- to NH_4^+, via N_2O. NAR is a membrane-bound nitrate reductase system expressed under high nitrate concentrations, converting NO_3^- to NO_2^-, while NAP is the periplasmic nitrate reductase system expressed under nitrate-limiting conditions. The periplasmic location of this enzyme suggests that NapA could carry out the first step in nitrate reduction and detoxification

1.6.1 Cytochrome *c* nitrite reductase

Cytochrome *c* nitrite reductase (NrfA) is a structurally and spectropotentiometrically characterized enzyme (Bamford, Angove et al. 2002; Burlat, Gwyer et al. 2005; Clarke, Kemp et al. 2008), which catalyses the reduction of nitrite to ammonia. In *E. coli*, NrfA has also been shown to reduce, and consequently detoxify, NO (Poock, Leach et al. 2002; van Wonderen, Burlat et al. 2008). In the related enteric pathogen *Campylobacter jejuni*, HmpA and NorV are absent, (Pittman, Elvers et al. 2007), although *C. jejuni* possesses a single domain globin, Cgb, which is inducible by, and protects against, nitrosative stress (Elvers, Turner et al. 2005).

NrfA, positioned in the periplasm of the bacterial cell, is well placed to carry out the detoxification of NO before NO enters the cell. NrfA may provide the first line of defence in protection against RNIs (Pittman, Elvers et al. 2007). However, it has been shown that the presence of oxygen inhibits NrfA activity (Wang and Gunsalus 2000; Poock, Leach et al. 2002), suggesting that any potential role for NrfA exists under anoxic or micro-oxic environmental conditions.

A study with *E. coli* NrfA using protein film voltammetry has unambiguously demonstrated that purified NrfA has a genuine NO reductase activity (van Wonderen, Burlat et al. 2008). At pH 7, the K_m of Nrf for NO is around 300 µM, which compares to around 10 µM for nitrite. It should be noted though that the NrfA k_{cat} is very high. It can turnover NO at around 5000 e s^{-1}, giving a catalytic efficiency (k_{cat}/K_m) of ~2x10^6 $M^{-1}s^{-1}$, which is an order of magnitude higher than that of flavorubredoxin (40x10^6 M^{-1} s^{-1}) (Gardner, Helmick et al. 2002; Gomes, Giuffre et al. 2002; van Wonderen, Burlat et al. 2008). Thus even at NO concentrations well below the K_m, turnover of NO by NrfA could be fast. For example, at the 10 µM NO levels produced extracellularly by activated

macrophages (Raines, Kang et al. 2006), it has been estimated that NrfA will reduce 27 NO s^{-1} (van Wonderen, Burlat et al. 2008).

Given the higher K_m for NO than nitrite it is plausible that important roles for NrfA as an NO reductase might occur in environments in which the NO concentration is higher than the nitrite concentration. Such environments might be found in the gastrointestinal tract where the acidic conditions of the stomach lead to nitrite disproportionating to a range of species, including NO(Equation 1). Since NrfA is a periplasmic protein its activity will be influenced strongly by the extracellular pH. However, NrfA is most active as an NO reductase at acid pH with activity demonstrated at pH3 (van Wonderen, Burlat et al. 2008), which would suggest that it would be active in a micro-oxic, acidic, NO-containing stomach environment.

As well as being exposed to exogenous NO, *Salmonella* also produces NO, when exposed to increased levels of nitrite. It is interesting that in *Salmonella*, though not in *E. coli*, both NrfA and NirB have been excluded as candidates for this NO production; however, the membrane-bound nitrate reductase NarGHJI seems to be responsible for nitrite-induced NO production (Gilberthorpe and Poole 2008). Vasodilation and disruption of the tight junctions between epithelial cells are two effects of NO in the gut (Resta-Lenert, Smitham et al. 2005). It could be to the advantage of *Salmonella* to produce NO to cause these effects, to facilitate easier invasion of the epithelium, and access to sub-epithelial tissues. In addition, NO produced by *Salmonella* may kill other competing microbial populations in the gut, while *Salmonella* itself is capable of detoxifying the NO through various pathways involving Nrf as already described, Nor and Hmp.

1.6.2 Flavorubredoxin

Flavorubredoxin (NorV) is a nitric oxide reductase which reduces NO to N_2O in anaerobic conditions (Gardner, Helmick et al. 2002; Hutchings, Mandhana et al. 2002). NorV has been extensively studied in *E. coli*, and work on other proteobacteria, including *S*. Typhimurium, has demonstrated that both NorV and NorR are largely conserved (Tucker, D'Autreaux et al. 2004). NorR senses NO levels, and activates transcription of *norV*. In *E. coli*, activity of NorV contributes to bacterial resistance to nitrosative killing (Gardner, Helmick et al. 2002; Hutchings, Mandhana et al. 2002). However, NorV on its own is not required for *E. coli* survival in mouse macrophages (Pullan, Gidley et al. 2007) and is not required for *Salmonella* survival in mice (Bang, Liu et al. 2006). In a *norR* mutant of *E. coli*, NO is not reduced to N_2O rapidly enough to prevent formation of nitrite. In cultures of *E. coli norR* mutants, *nrfA* expression is induced in response to the increase in nitrite, which combined with the continuing activity of HmpA (see below), would mask the loss of NorV, and could explain the lack of attenuation of the *norV* mutant in murine macrophages (Pullan, Gidley et al. 2007).

1.6.3 Flavohaemoglobin

Flavohaemoglobin (HmpA) is an enzyme containing an N-terminal haem group and a C-terminal reductase; it has been suggested that HmpA may play a role in NO detoxification in both aerobic and anaerobic environments (Mills, Rowley et al. 2008). It is clear that in both *Salmonella* and *E. coli*, transcription of the *hmpA* gene is activated by the addition of NO (Poole, Anjum et al. 1996; Crawford and Goldberg 1998). In *E. coli*, the free radical nitric oxide is dioxygenated by HmpA to produce nitrate (Gardner, Gardner et al. 1998; Hausladen, Gow et al. 1998; Gardner 2005).Expression of *hmpA* in *Salmonella*

has been shown to be induced by NO, and repressed by intracellular iron, an important mechanism by which detoxification of NO is accomplished without causing oxidative stress (Bang, Liu et al. 2006). Such NO detoxification is vital for the survival of both oxidative and nitrosative stresses. A key regulator of HmpA expression is NsrR, which encodes a NO sensitive regulator (Bodenmiller and Spiro 2006). Purified NsrR from *E. coli* has been experimentally confirmed to contain an NO sensitive iron-sulphur cluster which is vital for effective DNA binding activity (Tucker, Hicks et al. 2008). Other recent work has demonstrated that *hmp* expression is repressed in the absence of NO by NsrR, resulting in protection of *S.* Typhimurium *in vitro* and in macrophages, from the effects of both oxidative and nitrosative stress (Gilberthorpe, Lee et al. 2007). Troxell *et al* have also recently demonstrated that HmpA is repressed by the Fur regulon in *Salmonella*, which is responsible for iron homeostasis in bacteria as a Ferric Uptake Regulator (Troxell, Fink et al. 2011). The work of Troxell *et al* also reinforces the link between Fur regulation and H-NS repressed genes.

Other work reveals that *hmpA* is highly induced in *S*. Typhimurium inside macrophages (Eriksson, Lucchini et al. 2003), suggesting that HmpA is involved in the bacterial defence against nitrosative burst. However, this is not the case in epithelial cells, where *Salmonella* is not exposed to the oxidative or nitrosative killing mechanisms (Hautefort, Thompson et al. 2007). *S*. Typhi also up-regulates HmpA production inside human macrophages (Faucher, Porwollik et al. 2006), and a *S.* Typhimurium *hmpA* mutant is attenuated in human macrophages (Stevanin, Poole et al. 2002). Other work reports that *hmpA* mutants of the *S.* Typhimurium strain 14028 are attenuated in a C3H/HeN mouse virulence model (ity^R – genetically resistant to *Salmonella* infection), but not in C57/BL6 mice (ity^S– genetically susceptible to *Salmonella* infection) (Bang, Liu et al. 2006). C57/BL6 mice succumb to *S.* Typhimurium infection before they produce a nitrosative burst. *Candida albicans* also uses two domain

flavohaemoglobins which are similar to Hmp which may explain at least in part how *C. albicans* overcomes the presence of otherwise toxic salivary nitrogen species when initiating oropharyngeal infections (Poole, Anjum et al. 1996).

It is clear that NrfA, NorV, and HmpA play crucial roles in the detoxification of NO in anaerobiosis by *Salmonella* (Kim, Orii et al. 1999). Mills *et al.* (2008) constructed all possible combinations of *norV*, *nrfA* and *hmpA* single, double and triple mutants. Addition of NO to cultures of all single mutants and wild type strains caused a temporary growth arrest. In wild type, *hmpA* and *nrfA* single mutants, the growth rate recovered after a similar interval, but in the *norV* mutant, this recovery only occurred after a significant delay (Mills, Rowley et al. 2008). This suggests an important role for NorV in anaerobic NO detoxification. However, as the *norV* mutant strain eventually recovered from the addition of NO, another enzyme, possibly NrfA or HmpA, must be responsible for detoxification of the NO in the absence of NorV. Double mutants revealed that the most severe growth arrest occurred in the *ΔnrfAΔnorV* double mutant (Mills, Rowley et al. 2008). The continued presence of HmpA in this mutant was unable to compensate for the loss of NrfA and NorV, under anoxic conditions, which was consistent with some earlier work using *E. coli* (Gardner and Gardner 2002), but contrary to previous findings in *Salmonella* (Crawford and Goldberg 1998). Therefore the most important enzymes for continued growth of *Salmonella* in anoxic minimal glucose media after exposure to NO are NrfA and NorV. HmpA was shown to have a minor role in NO detoxification under anoxia, but a more important role in aerobic conditions (Mills, Rowley et al. 2008).

Although a *Salmonella* strain that lacks NrfA, NorV and HmpA is hypersensitive to killing by NO *in vitro*, and is significantly more sensitive to NO than any of the single mutants, this same triple mutant is only partially attenuated during infection of cultivated murine macrophages or following intraperitoneal infection

of C3H/HeN mice (G. Rowley, unpublished data). These findings suggest that other mechanisms for NO detoxification exist which are induced during animal infection.

As discussed previously, various nitrogen species are present in the gastrointestinal tract, which is also a largely anaerobic environment in mammals (Backhed, Ley et al. 2005). However, a microaerobic niche is potentially vital for successful occupation by *E. coli* in competitive environments (Jones, Chowdhury et al. 2007), challenging the established view of the gut as an exclusively anaerobic environment. Still, it is likely that *Salmonella* is exposed to NO, nitrate or nitrite, or all three, which must be detoxified to allow *Salmonella* to survive in the lumen of the gastrointestinal tract (Xu, Xu et al. 2001; Lundberg, Weitzberg et al. 2004). Figure 5 outlines the activities and functionalities of various NO detoxification enzymes in enteric bacteria. The NO detoxification mechanisms which *Salmonella* uses in the luminal environment of the gastrointestinal tract remain to be fully explored and understood. In contributing to this understanding, Bourret *et al* have elegantly demonstrated that nitric oxide prevents production of a *de novo* ATR by *S*. Typhimurium (Bourret, Porwollik et al. 2008). The authors propose that this suppression of the ATR contributes to the antimicrobial activity of nitrogen oxides, and have shown that this impacts both on oral virulence and faecal shedding of *S*. Typhimurium. It is interesting to note that induction of the ATR increases the vulnerability of *Salmonella* to succumb to oxidative stress (Greenacre, Lucchini et al. 2006).

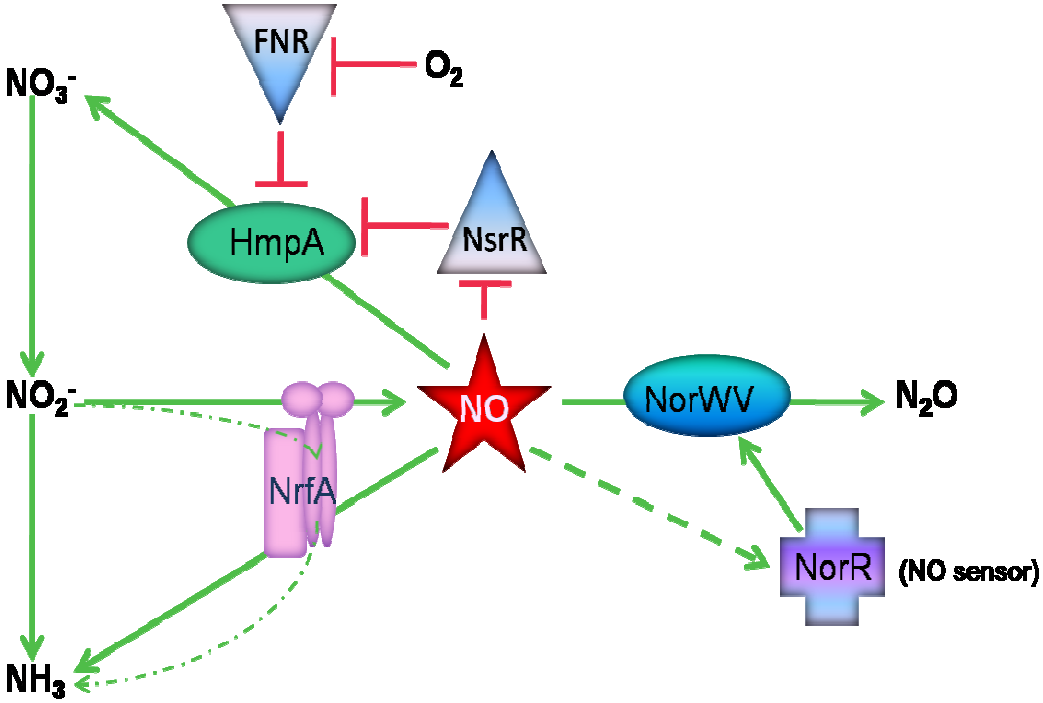


Figure 5 – Involvement of enzymes in nitric oxide detoxification in enteric bacteria. Detoxification of NO is achieved through involvement of several enzymes. NrfA, a cytochrome *c* nitrite reductase (*nrfA*), reduces either nitrite or NO to ammonium; a flavorubredoxin (*norV*) reduces NO to nitrous oxide (N_2O) in either anaerobic or low oxygen conditions; HmpA, a flavohemoglobin, (*hmpA*), works in oxic environments to oxidize NO to nitrate, or in anoxic conditions HmpA reduces NO to N_2O. It should be noted that NO produced by NrfA is predominantly membrane-bound.

1.7 NR-A, Nap and NR-Z nitrate reductase systems

Besides NO detoxification, bacteria have developed additional enzymatic systems, mainly nitrate reductases, in their respiratory pathways to deal with other compounds such as nitrate. *S*. Typhimurium possesses three distinct nitrate reductase systems, which are the NarGHJI membrane-bound system, the periplasmic Nap nitrate reductase and the NarZYWV isozyme of NarGHJI, as described earlier. The extent of any involvement of these three nitrate reductase systems in *Salmonella* infection remains to be fully understood.

1.7.1 NR-A

NR-A is the structurally defined NarGHJI membrane-bound nitrate reductase system expressed under high nitrate concentrations in anaerobiosis in *E. coli* (Stewart and Berg 1988; Jormakka, Richardson et al. 2004; Bertero, Rothery et al. 2005). Structurally, the enzyme comprises three subunits. The catalytic site (NarG) is a large molybdoprotein, with smaller iron-sulphur (NarH) and cytochrome *b* (NarI)subunits (Neidhardt 1996). NarJ is a chaperone protein vital to the assembly of this enzyme (Sodergren, Hsu et al. 1988; Blasco, Dos Santos et al. 1998). The active site for nitrate reduction, the NarG and NarH subunits, faces into the cytoplasm of the bacterial cell; the quinol oxidation occurs at the NarI subunit with accompanying release of protons to the periplasm (Sodergren, Hsu et al. 1988), while nitrate reduction is carried out at the NarG site with consumption of protons from the cytoplasm (Bertero, Rothery et al. 2003). When the NR-A operon is fully activated, it carries out 98% of the nitrate reductase activity in *E. coli*, under the regulation of FNR in anaerobiosis, and NarL-Phosphate in nitrate-rich conditions (Bonnefoy and Demoss 1994). In *Pseudomonas aeruginosa*, the *narK1K2GHJI* operon encodes two nitrate/nitrite transporters and a nitrate reductase (Schreiber, Krieger et al. 2007). Other pathogenic microbes also use similar transport and reductase systems, although regulation may differ between species (Fedtke, Kamps et al. 2002). Expression of the *Mycobacterium tuberculosis narGHJI* genes in a nitrate reductase *E. coli* mutant allowed anaerobic growth in the presence of nitrate (Sohaskey and Wayne 2003). This has been further refined to show that a *M. tuberculosis narG* mutant is unable to grow on nitrate, with the conclusion that NarGHJI is vital to *M. tuberculosis* in the assimilatory reduction of nitrate (Malm, Tiffert et al. 2009).Expression of *narG* has been shown to be under strict positive control by FNR in *E. coli* (Ansaldi, Theraulaz et al. 2007). Recent work has concluded that

NarGHJI is responsible for NO production in *Salmonella*, in response to high levels of nitrite (Gilberthorpe and Poole 2008).This has been explored further by Rowley *et al* who have reported that it is a combination of sufficiency of nitrate, accumulation of nitrite and activity of the NarGHJI membrane-bound nitrate reductase in *Salmonella* which results in production of both nitrous oxide (N_2O) and NO (Rowley, Hensen et al. 2012). It is interesting that the NarL transcriptional regulator component which lies upstream of the NarGHJI operon has been shown to be controlled by Fur in *Salmonella enterica* (Teixido, Cortes et al. 2010). Fur is a metalloregulator of Fe^{2+} cations within the cell, and a global regulator due to its involvement in both iron uptake and control of nitrate respiration due to the ability to sense the redox status within the cell. Other recent work has also confirmed that Fur exerts a negative regulatory effect on HmpA (Troxell, Fink et al. 2011).

1.7.2 Nap

Nap is the recently identified periplasmic nitrate reductase system where NapA is the catalytic enzyme, which in *E. coli* is expressed under nitrate limiting conditions. While this enzyme has been well explored in other bacterial species, such as *Paracoccus denitrificans* (*P. denitrificans*), previously known as *Thiosphaera pantotropha* (Berks, Richardson et al. 1995; Sears, Bennett et al. 1995), in *Salmonella*, much work remains to be done. The Nap system has been characterized structurally and potentiometrically in *E. coli* (Jepson, Mohan et al. 2007), using methods including crystallography, X-ray electrochemistry, and electron spin resonance spectroscopy. The large subunit (NapA) comprises a molybdoprotein with an iron-sulphur cluster (Breton, Berks et al. 1994), while the NapB subunit is the cytochrome *b* component of this enzyme (Berks, Richardson et al. 1994; Berks, Richardson et al. 1995). There is an additional cytochrome *c* encoded by the NapC subunit in *E. coli* and *Salmonella* (Bamford, Angove et al.

2002), although in several species (*Wolinella succinogenes*, *Campylobacter jejuni*, *Shewanella oneidensis*), this component is not an integral part of the Nap operon (Simon, Sanger et al. 2003). Additional polypeptides are encoded by NapD and NapE subunits; it has been suggested that NapD plays a role in the maturation of NapA (Potter and Cole 1999; Brige, Leys et al. 2002). The precise function of NapE is, as yet, unknown, but has been predicted simply as an integral membrane protein (Simon, Sanger et al. 2003). Recent work suggests that it is possible for different arrangements of the subunits for the Nap operon to reflect the requirement for physiologically different processes depending on the environmental conditions which prevail (Simpson, Richardson et al. 2010), at least in the highly adaptive organism *Shewanella*.The periplasmic location of this enzyme suggests that NapA carries out the first step in nitrate reduction and detoxification. Nap is repressed in the presence of oxygen in denitrification and dissimilation to ammonia (nitrate ammonification) (Cruz-Garcia, Murray et al. 2007). The genome sequence of *E. coli* revealed the Nap structural genes *napFDAGHBC* (Stewart, Lu et al. 2002). It may be that the Nap reductase, which has a higher affinity for nitrate than NR-A, is anaerobically induced when nitrate is limited, while NR-A is induced by a high concentration of nitrate (Potter, Millington et al. 1999; Constantinidou, Hobman et al. 2006). This has been borne out by recent work with *Salmonella* by Rowley *et al* (Rowley, Hensen et al. 2012). In *Shewanella*, at least, regulation of Nap expression has been attributed to the oxygen responsive fumarate nitrate regulator Fnr, and the cAMP receptor protein (Crp) (Simpson, Richardson et al. 2010). Previously, it has also been shown (Stewart, Bledsoe et al. 2009) that in anaerobic and nitrate limited conditions, transcription of napF is also regulated by Fnr. In addition, the same work concluded that in *E. coli*, napF expression could be stimulated under energy limitation by Crp (Stewart, Bledsoe et al. 2009).

1.7.3 NR-Z

It had been noted that *narG* mutants still displayed some residual nitrate reductase activity (Barrett and Riggs 1982; Iobbi, Santini et al. 1987), supporting the suggestion that a second nitrate reductase in addition to the NR-A system existed. NR-Z, encoded by *narZYWV*, is an isozyme of the membrane-bound nitrate reductase enzyme encoded by the *narGHJI* operon (Blasco, Iobbi et al. 1990), and also comprises three subunits (Blasco, Pommier et al. 1992). The transcription unit for NR-Z is 73% homologous to that for NR-A, leading to the hypothesis that the presence of NR-Z in the *E. coli* genome has arisen due to duplication of a DNA fragment larger than the *narGHJI* operon, but including the components of the *narGHJI* operon. Nevertheless, the *narZYWV* (NR-Z)and *narGHJI* (NR-A) operons are regulated differently, and it has been suggested that expression of NR-Z is stress-related in *Salmonella* (Spector, Garcia del Portillo et al. 1999). The *narZYWV* operon is constitutively expressed in *E. coli* (Bonnefoy and Demoss 1994). Under aerobic conditions, the only anaerobic respiratory system shown to be induced is the nitrate reductase NR-Z, controlled by the alternative sigma factor RpoS, and expressed during the stationary growth phase (Chang, Wei et al. 1999; Rychlik and Barrow 2005; Clegg, Jia et al. 2006; Jones, Chowdhury et al. 2007).

1.8 Summary and context

Salmonella and other pathogens have clearly developed several overlapping or complementary systems to avoid the innate immune response and killing by NO. *Helicobacter pylori* have the capacity to inhibit the production of NO by eukaryotic host macrophage cells (Gobert, McGee et al. 2001). *H. pylori* inhibits macrophage NO production by competing with host cell iNOS for the available L-

arginine used by NOS to produce NO. However, little is known about how entero-pathogens such as *Salmonella* adapt to the biochemical environment of the mammalian gastrointestinal tract during the early stage of infection. Given the largely anoxic nature of the gastrointestinal tract, the ability of nitrate to serve as a terminal electron acceptor during anaerobic bacterial growth, and the relatively high levels of nitrate, it is possible that there is a connection between the process of bacterial infection, occupation of the gastrointestinal tract and nitrate dissimilation by *Salmonella*. The role of the three nitrate reductase systems of *Salmonella*, NR-A, Nap and NR-Z, has not yet been elucidated, and needs to be established in the successive environmental adaptations of *Salmonella* which must take place in order for successful infection, colonization and invasion to occur.

Undoubtedly, further work to elucidate the role of NO detoxification by NorV, NrfA and HmpA in the growth and/or survival of *Salmonella* in the gastrointestinal tract is indicated. It is possible that other factors are involved in the survival of *Salmonella* in the gastrointestinal tract, not only in the detoxification of RNIs, but also by the dissimilation of nitrate. The functions and roles of various components of nitrate reductase systems are under investigation in our laboratory. An understanding of the precise role of different aspects of nitrogen metabolism and detoxification during infection could lead to the elucidation of novel opportunities and potential targets for the development of therapeutic approaches or vaccines.

As described above, many virulence determinants for *Salmonella* have been identified and studied, but little attention has been paid to some aspects of *Salmonella* metabolism in relation to virulence (Darwin and Miller 1999; Wallis and Galyov 2000; Zhang, Kingsley et al. 2003).*Salmonella* is exposed to a range of reactive nitrogen species, during both the enteric and systemic phases of

infection. *Salmonella* Typhimurium has three distinct nitrate reductase systems, NR-A, Nap and NR-Z, as outlined above.

Since (i) the gastrointestinal tract is largely anaerobic, albeit with microaerobic niches (Jones, Chowdhury et al. 2007), (ii) nitrate can serve as a terminal electron acceptor during anaerobic bacterial growth (Lundberg, Weitzberg et al. 2004), and (iii) there are relatively high levels of nitrate in the mammalian gut, perhaps bacterial infection, colonisation of the mammalian gastrointestinal tract and nitrate dissimilation are connected.

The possibility that a link exists between nitrate, NO dissimilation and detoxification and gut occupation by *Salmonella* is an interesting one, and this project provides the first integrated view of the importance of nitrate reduction on gut colonization by an enteric pathogen. In the long-term, this will lead to the development of new strategies to defend the host against *Salmonella* infection.

With changes between aerobic and anaerobic growth, there are also differences in the carbon substrates which are used for growth. Each of these substrates has a specific route which enables it to be catabolised and converted to energy, which in turn requires a specific set of enzymes. In the *in vitro* work in this study, *Salmonella* was grown and challenged aerobically and anaerobically using glucose and glycerol as different carbon sources, to investigate and identify any differences between some of the enzymes involved in nitrogen species as electron acceptors.

The role of the three nitrate reductase systems in *Salmonella* infection will be assessed. For example, the *nap* operon encodes periplasmic nitrate reductase, and related proteins, that make up one of the three nitrate reductase systems of *Salmonella*, outlined previously. A *nap* operon deletion mutant has previously been tested for its ability to invade epithelial cells, and shows a 20-30% attenuation of invasive capability compared with SL1344 wild type (Alston *et al*., unpublished data), suggesting that the *nap* operon could play a role in the early

process of *Salmonella* infection. Other work on *S*. Typhi has found that mutants which were unable to use either nitrate or fumarate as their terminal electron acceptor were less invasive in epithelial cells than wild type (Contreras, Toro et al. 1997), leading this team to conclude that anaerobic respiration is a requirement to ensure successful invasion for *S*. Typhi.

Targets for novel therapeutics or vaccines may be identified through development of an integrated view of any link between nitrate reduction and gut colonisation by enteric pathogens.

Chapter 2 - Materials and Methods

2.1 Bacterial strains and culture conditions

The bacterial strains used in this study are derived from *Salmonella* Typhimurium wild type strain SL1344 (Hoiseth and Stocker 1981; Wray and Sojka 1984), and shown in Table 1.

Table 1 - Strains and Plasmids used in this study

Strain	Genotype	Reference
SL1344	*S.*Typhimurium WT, His^-	Hoiseth and Stocker, 1981
JH3523	*S.* TyphimuriumSL1344 Δ*narZYWV*::*kan*	This study
JH3524	*S.* TyphimuriumSL1344 Δ*narZ*::*kan*	This study
JH3525	*S.* TyphimuriumSL1344 Δ*narWV*::*kan*	This study
JH3526	*S.* TyphimuriumSL1344 Δ*narGHJI*::*kan*	This study
JH3527	*S.* TyphimuriumSL1344 Δ*narG*::*kan*	This study
JH3528	*S.* TyphimuriumSL1344 Δ*napDA*::*kan*	This study
JH3529	*S.* Typhimurium SL1344 Δ*narZYWV*::*cat*	This study
JH3530	*S.* TyphimuriumSL1344 Δ*narZ*::*cat*	This study
JH3531	*S.* Typhimurium SL1344 Δ*narWV*::*cat*	This study
JH3532	*S.* TyphimuriumSL1344 Δ*narGHJI*::*cat*	This study
JH3533	*S.* TyphimuriumSL1344 Δ*narG*::*cat*	This study
JH3534	*S.* TyphimuriumSL1344 Δ*napDA*::*cat*	This study
JH3553	*S.* TyphimuriumSL1344 *ΔnarG::cat ΔnapDA::kan*	This study
JH3554	*S.* TyphimuriumSL1344 *ΔnarWV::cat ΔnapDA::kan*	This study
JH3555	*S.* Typhimurium SL1344 *ΔnarWV::cat ΔnarGHJI::kan*	This study
JH3556	*S.* TyphimuriumSL1344 *ΔnarZ::cat ΔnarGHJI::kan*	This study
JH3557	*S.* Typhimurium SL1344 *ΔnarZ::cat ΔnarG::kan*	This study
JH3558	*S.* TyphimuriumSL1344 *ΔnarZYWV::cat ΔnapDA::kan*	This study
JH3559	*S.* TyphimuriumSL1344 *ΔnarZYWV::cat ΔnarG::kan*	This study
JH3560	*S.* TyphimuriumSL1344 *ΔnarZ::cat ΔnapDA::kan*	This study

Strain	Genotype	Reference
JH3561	*S.* TyphimuriumSL1344 *ΔnarWV::cat ΔnarG::kan*	This study
JH3562	*S.* TyphimuriumSL1344 *ΔnarGHJI::cat ΔnapDA::kan*	This study
JH3563	*S.* TyphimuriumSL1344 *ΔnarZYWV::cat ΔnarGHJI::kan*	This study
JH3564	*S.* Typhimurium SL1344 *ΔnarZ::cat ΔnarWV::kan*	This study
GR0281	*S.* TyphimuriumSL1344 *ΔnarZYWV::kan ΔnarGHJI::cat ΔnapDA*	This study
MG1655	*E. coli*K12	
	*E. coli*BW25141/pKD3 CGSC# 7631::cat	Datsenko & Wanner, 2000
	*E. coli*BW25141/pKD4 CGSC# 7632::kan	Datsenko & Wanner, 2000
	*E. coli*BW25113/pKD46 CGSC# 7630::amp	Datsenko & Wanner, 2000
	S. Typhimurium LT2A(pCP20)::amp	Datsenko & Wanner, 2000
	S. TyphimuriumSL1344 Wild Type:pMP220	This study
	S. TyphimuriumSL1344*ΔhilA*:pMP220	This study
	S. TyphimuriumSL1344*Δcrp*:pMP220	This study
	S. TyphimuriumSL1344*Δfnr*:pMP220	This study
	S. TyphimuriumSL1344*ΔrpoS*:pMP220	This study
	S. TyphimuriumSL1344*ΔSO1(SPI-1)*:pMP220	This study
	S. TyphimuriumSL1344*ΔfisA*:pMP220	This study
	S. TyphimuriumSL1344 Wild Type:pMP220:*napF*	This study
	S. TyphimuriumSL1344*ΔhilA*:pMP220:*napF*	This study
	S. TyphimuriumSL1344*Δcrp*:pMP220:*napF*	This study
	S. TyphimuriumSL1344*Δfnr*:pMP220:*napF*	This study
	S. TyphimuriumSL1344*ΔrpoS*:pMP220:*napF*	This study
	S. TyphimuriumSL1344*ΔSO1(SPI-1)*:pMP220:*napF*	This study
	S. TyphimuriumSL1344*ΔfisA*:pMP220:*napF*	This study
	S. TyphimuriumSL1344 Wild Type:pMP220:*narG*	This study
	S. TyphimuriumSL1344*ΔhilA*:pMP220:*narG*	This study

Strain	Genotype	Reference
	S. TyphimuriumSL1344*Δcrp*:pMP220:*narG*	This study
	S. TyphimuriumSL1344*Δfnr*:pMP220:*narG*	This study
	S. TyphimuriumSL1344*ΔrpoS*:pMP220:*narG*	This study
	S. TyphimuriumSL1344*ΔSO1(SPI-1)*:pMP220:*narG*	This study
	S. TyphimuriumSL1344*ΔfisA*:pMP220:*narG*	This study

Plasmid	Genotype	Reference
pKD3	Template plasmid: pANT-S *γ*derivative Amp^R, FRT-flanked cat^R gene	(Datsenko and Wanner 2000)
pKD4	Template plasmid: pANT-S *γ* derivative Amp^R, FRT-flanked kan^R gene	(Datsenko and Wanner 2000)
pKD46	pINT-ts derivative (Ts-), $araC^+$ P_{BAD} (λβ*exo* DNA fragments), Amp^R	(Datsenko and Wanner 2000)
pCP20	bla cat pSC101 oriTS *FLP-, λ* $cI857^+$,$λp_R$ Rep^{ts}, Amp^R, Cat^R	(Cherepanov and Wackernagel 1995)
pMP220	IncP vector with promoterless *lacZ*, reporter plasmid, Tet^R	(Spaink 1987)

The following media was used for culturing bacterial strains:

- Luria-Bertani (LB) broth, (Sambrook and Russell 2001);
  - 10g Bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1L water, pH adjusted to 7.2, and autoclaved at 121℃
- LBS (modified LB with 0.3M NaCl final concentration);
  - 10g Bacto-tryptone, 5g bacto-yeast extract, 20g NaCl in 1L water, pH adjusted to 7.2, and autoclaved at 121℃
- M9 broth (Sambrook and Russell 2001);
  - 100mL 5xM9 salts, 1mL 1M $MgSO_4$, 10mL 20% Glucose and 500µL 1M $CaCl_2$, all sterilised solutions, in 375mL pre-autoclaved water
- Lennox broth (Sambrook and Russell 2001);
  - 10g Bacto-tryptone, 5g bacto-yeast extract, 5g NaCl in 1L water, pH adjusted to 7.2, and autoclaved at 121℃

- Xylose-Lysine-Desoxycholate (XLD) (Oxoid, product code CM0469);
- non-lysogenic P22 transductants were screened on green plates (Bochner 1984);
- Minimal Glucose Medium (MGM) (Mills, Rowley et al. 2008);
    - Per litre
        - KH_2PO_4 2.25g
        - K_2HPO_4 5.25g
        - Ammonium Sulphate 1g
        - Sodium Citrate 0.5g
        - Magnesium Sulphate 0.05g
        - $((NH_4)6Mo_7)24$ 1mL of 1mM solution (0.024g in 20mL water)
        - Na_2SeO_4 1mL of 1mM solution (0.003g in 20mL water
        - Magnesium Chloride 0.25mL of 30% solution
        - Manganese Chloride 0.25mL of 4% solution
        - Calcium Chloride 0.25mL of 0.51% solution
        - Iron Chloride 0,25mL of 1.85% solution

    Autoclaved at 121℃, and just prior to use, the addit ion of:

        - Glucose 50mL of 20% solution
        - Casamino Acids 1mL of 10% solution

- Minimal Glycerol Nitrate (Fumarate) Medium (GN/GNF) (Mills, Rowley et al. 2008);
    - Per litre
        - KH_2PO_4 2.25g
        - K_2HPO_4 5.25g

- Ammonium Sulphate 1g
- Sodium Citrate 0.5g
- Magnesium Sulphate 0.05g
- $((NH_4)6Mo_7)24$ 1mL of 1mM solution (0.024g in 20mL water)
- Na_2SeO_4 1mL of 1mM solution (0.003g in 20mL water
- Magnesium Chloride 0.25mL of 30% solution
- Manganese Chloride 0.25mL of 4% solution
- Calcium Chloride 0.25mL of 0.51% solution
- Iron Chloride 0,25mL of 1.85% solution
- Sodium Nitrate 1.9g

Autoclaved at 121℃, and just prior to use, the addit ion of:

- Glycerol 20mL of 2M solution
- (Sodium Fumarate 40mL of 1M solution
- Casamino Acids 1mL of 10% solution

Ampicillin and Streptomycin antibiotics were used at 100µg/ml, Chloramphenicol at 10µg/ml, Kanamycin and Tetracyclin at 50µg/ml final concentration.

2.2 Storage of Strains

Long term storage of the mutants constructed was carried out by picking a single colony to set up an overnight culture in 5ml LB supplemented with the appropriate antibiotic, incubated at the appropriate temperature (30℃ or 37℃). The culture was frozen down (930µl culture + 70µl Dimethyl Sulfoxide (DMSO) as a cryoprotectant) in 1ml cryotubes, at -80℃.

2.3 DNA Isolation

2.3.1 Isolation of Bacterial Chromosomal and Plasmid DNA

Rapid isolation of chromosomal and plasmid DNA for use as templates in polymerase chain reactions (PCR) was achieved by using a boilate method. A single colony was picked from a fresh plate and mixed in 200µl sterile water. This was boiled for five minutes at 100℃, centrifuged at 5000rpm in a microcentrifuge. A 2µl aliquot of the supernatant was used in a 50µl PCR reaction.

2.3.2 Plasmid DNA purification

Briefly, strains were grown overnight at 30℃, for pK D46 and pCP20, or 37℃ for pKD3, pKD4 and pMP220, in LB broth containing the appropriate antibiotic. DNA purification was carried out using kits from QIAgen, according to their protocol, and based on the alkaline lysis method (Sambrook and Russell 2001). Purified plasmids were checked by agarose gel electrophoresis (0.8% agarose in 1xTAE with 0.5µg/ml ethidium bromide).

2.4 Transfer of DNA

2.4.1 Electroporation of Bacterial Cells

Electrocompetent cells were prepared for the routine transformation of plasmid DNA and ligation reactions and also for mutant construction. Based on the technique described by Datsenko and Wanner, an overnight culture of the recipient strain of interest was diluted 100-fold in 25ml LB broth and cultivated until it reached a ΔOD_{600nm} of between 0.6 and 1.0 (Datsenko and Wanner

2000). For gene deletion mutant construction, the cultures were carried out in Lennox broth containing 10mM L-Arabinose at 30℃. The culture was immediately stopped on ice. Bacterial cells were harvested by centrifugation for 5 minutes at 8000rpm at 4℃. The supernatant was removed and cells were washed three times in 20ml of sterile ice-cold 10% glycerol to remove excess salts from the growth medium. The electrocompetent cells were subsequently concentrated 100-fold in 10% glycerol and kept on ice until further processing. For long term storage, the cells were aliquoted by 50µl and snap frozen in a mixture of dry ice and ethanol, and stored at -80℃.

To 50µl of electrocompetent cells was added 500ng to 1µg of the plasmid or PCR product. This cell/DNA mix was transferred to ice cold electroporation cuvettes, and electroporated using a Life Technology electroporator (capacitance 330µF, low Ω, charge rate fast and 4KΩ), set to 2.5kV. Immediately following the electroporation, 1ml of pre-warmed broth was added to the electroporated cells and gently mixed by pipetting. Recovery of the cells was permitted at 30℃ (pKD46 or pCP20) or 37℃ (PCR products) for one hour. Aliquots (10µl, 100µl and 200µl) were spread plated on selective medium and incubated overnight at 30℃ (pKD46 or pCP20) or 37℃ (PCR product). A 'no DNA' negative control was set up for each experiment.

2.4.2 Transformation using chemically prepared competent *E. coli* cells

Chemically competent *E. coli* TOP10' cells (Invitrogen) were also used for some ligation reactions. A 5µl aliquot of DNA at a concentration of 1µg/µl from a ligation reaction was added to 200µl of the competent cells and left on ice for 30 minutes, then heat shocked at 42℃ for two minutes. They were immediately re-

incubated on ice for two minutes, and the 800µl of LB medium was added, and the cells were allowed to recover at 37℃ in aerobic con ditions. After a one hour recovery period, the aliquot was centrifuged at 13000rpm in a microcentrifuge, the supernatant was discarded and the cells were resuspended in 400µl fresh LB medium. The transformed cells were spread plated on selective medium and incubated at the appropriate temperature.

2.5 Mutagenesis

Mutations were constructed by one step inactivation of chromosomal genes in S. Typhimurium strain SL1344 by PCR products, using the Lambda-Red (λ-red) inactivation technique (Datsenko and Wanner 2000). The λ-red method uses three genes, γ (Gam), β (Bet), and exo (Exo), to inhibit host RecBCD exonuclease V (by Gam) allowing Bet and Exo to promote recombination by accessing DNA fragment ends. A representation of the method is visualised in Figure 6.

Chloramphenicol or kanamycin resistance cassettes were amplified by PCR from plasmids pKD3 and pKD4, respectively. Amplification was achieved by using primers listed in Table 2. The 5' end of each primer carried a 40 base pair flanking region homologous to the upstream or downstream regions of the operon or gene to be deleted, linked to a 3' region homologous to the antibiotic resistance cassette to be amplified. Each operon was to be replaced by both a kanamycin and a chloramphenicol resistance cassette to allow future combination of mutations. These PCR products were transformed by electroporation into the strain SL1344 harbouring the thermosensitive plasmid pKD46, which encodes the λ-red recombinase. This plasmid allows inducible expression (after addition of L-arabinose) of the recombination functions necessary for the gene replacement approach. Chloramphenicol and kanamycin

resistant colonies were purified on selective plates. Gene and operon deletions were subsequently transduced with P22 HT105/1 int-201phage (Schmieger 1972; Mann and Slauch 1997), back into a clean *S*. Typhimurium SL1344 background to avoid possible non-intentional recombination events (Gemski and Stocker 1967). PCR amplification of the mutated genome region was used to check for loss of the targeted gene. Mutagenesis was confirmed by using primers designed externally to the chromosomal loci where the desired mutation should lie, as well as primers designed specifically to the antibiotic cassette.

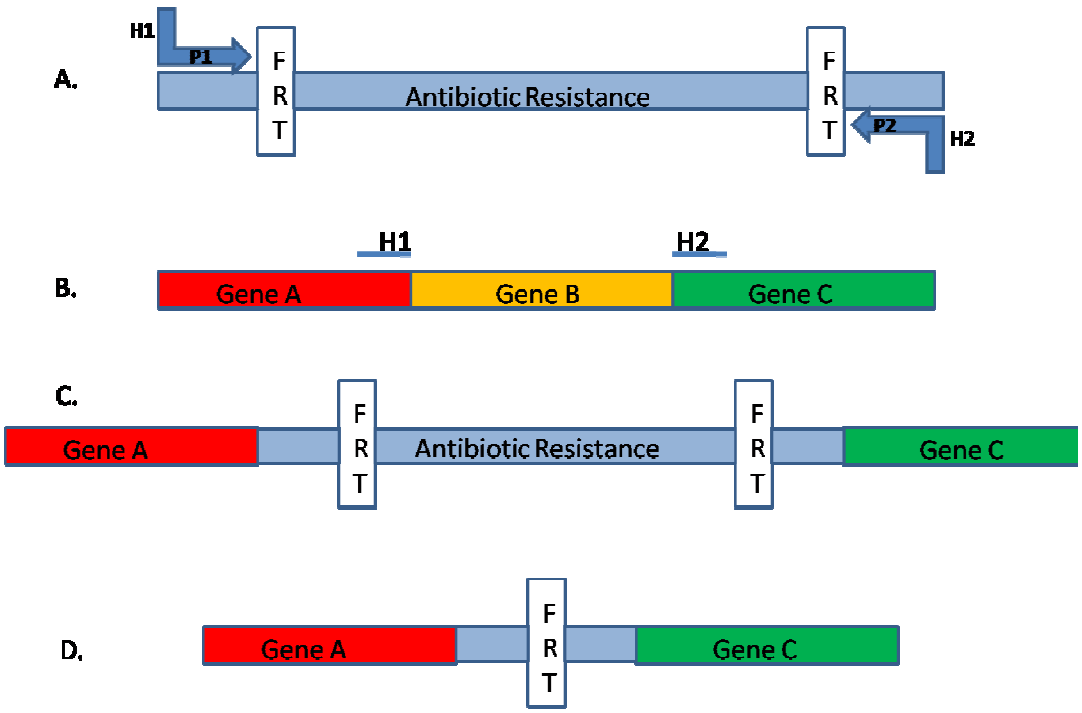


Figure 6 - **Lambda red one-step inactivation of genes**
H1 and H2 indicate the homology regions, whilst P1 and P2 identify the priming sites.
A. Chloramphenicol or kanamycin resistance cassettes were amplified by PCR from plasmids pKD3 and pKD4, respectively;
B. These PCR products were transformed by electroporation into the strain SL1344 harbouring the thermosensitive plasmid pKD46, which encodes the λ-red recombinase;
C. Chloramphenicol and kanamycin resistant colonies were purified on selective plates;
D. Incubation above 30℃ eradicated the temperature se nsitive pKD46, and mutants were subsequently transduced back into a clean *S*. Typhimurium SL1344 background to avoid unintended recombination events. Loss of the targeted gene was confirmed by PCR.

Table 2 – List of primers selected for specific deletion mutants

Oligonucleotide	Sequence (5' to 3')
nargredf	TTTATCAGAGAGCCGTAAGGTTCCACACAGGAGAAACCCGGTGTAGGCTGGAGCTGCTTC
nargredr	TTATCGAGATTCAGCACCATGCCGACTTGTGAACGAATTTCATATGAATATCCTCCTTAG
nariredr	AAAACTCCGCCGAAGCGGAGTTTAGGGCATCGAGAGAAAACATATGAATATCCTCCTTAG
narzredf	GACAGGATGGCGGAAAATTTATCGAAGCAGGAGAAATGTCGTGTAGGCTGGAGCTGCTTC
narzredr	TTATCGAGATTCAGTACCATCCCAACCTGTGAGCGTATTTCATATGAATATCCTCCTTAG
narvredr	TTTTTCTTTGCCCGGTAAGCGTATCCCCGGGCAAAGAGAACATATGAATATCCTCCTTAG
narwredf	TCAATATCACCGAAGTACGTGATAAAGCGGAGGGGGAATAGTGTAGGCTGGAGCTGCTTC
napdredf	GCTATCTGCCCCGTGTCAGCCATCAAAGCGGAGAACCACCGTGTAGGCTGGAGCTGCTTC
naparedr	GGCGGCCATTTTGGGGTTTCGCTGTACGGGACATAACGCGCATATGAATATCCTCCTTAG
nargextf nariextr	CGTTATCAATTCTCACGC AACGAAACAAGGCGGCGG
narzextf narvextr	CAACAATAACAACATACG TTCTCCTTTGACTGAAGA
narwextf narvextr	ACGGTTGCCACGGGTCGG TTCTCCTTTGACTGAAGA
nargextf nargextr	CGTTATCAATTCTCACGC CCTTCGCGACTGGTCCAG
narzextf narzextr	CAACAATAACAACATACG CCTTCACGTCCGGTCCAG
napdextf napaextr	TCCGGTATTTACCAGCCG GCGCCACGCCAACCGCCG
NarGpromF	GAATTCGCATTAACCGGTTC
NarGpromR	CTGCAGCGGGTTTCTCCTGT
NapFpromF	GAATTCTCTTCATTTGATTGT
NapFpromR	CTGCAGGATATCGCCCTTCC

2.5.1 P22 lysate preparation and transduction

Overnight cultures of the mutants of interest were diluted 100 fold in 5ml LB broth, and incubated at 37°C in aerated conditions (250 rpm) for an hour. 10µl of P22 HT105/1 int-201 SL1344 lysate was added to these cultures and incubated for a further five hours. Cells were subsequently lysed by addition of 500 µl $CHCl_3$. After at least one hour at 4°C, the lysates were centrifuged in order to separate phage particles and bacterial cell debris. The supernatant containing the lysate was transferred into clean tube and stored at 4°C after addition of few drops of $CHCl_3$ for long term storage. Transductions of the mutations were

carried out by addition of 10µl of the freshly prepared lysates to 90µl of an overnight culture of the recipient strain. After 45 minutes incubation at 37°C the mix was plated on selective agar plates. Non-lysogenic colonies were selected twice on Green Plates where they appear pale green. Finally, the mutations were confirmed by PCR to ensure that disruption of genes had been achieved using the external primers listed in Table 2.

2.5.2 Construction of double deletion mutants

Transductions of the double mutants were carried out by addition of 10µl of lysates to 200µl of an overnight culture of the recipient strain. After 60 minutes incubation at 37℃ the mix was plated on selective agar plates. Non-lysogenic colonies were selected twice on green plates, and finally, the mutations were confirmed by PCR, using the primers listed in Table 2.

2.5.3 Construction of triple deletion mutant

The pCP20 plasmid was used to flip out the resistance cassettes from single operon deletion mutants SL1344*ΔnarGHJI,* SL1344*ΔnarZYWV* and SL1344*ΔnapDA* (Ellermeier, Janakiraman et al. 2002). The pCP20 plasmid is temperature sensitive and encodes for the FLP recombinase (Cherepanov and Wackernagel 1995) which was required to 'flip' out the region of DNA between the FRT sites as illustrated and described in Figure 6 above. Strains containing either the chloramphenicol or kanamycin resistance cassettes were transformed with pCP20, recovered and plated on LB with appropriate antibiotic at 30℃. Resultant colonies were restreaked on LB with appropriate antibiotic at 30℃, and tested for loss of the resistance cassette by patching onto selective plates. Subsequently, culture at 37℃ prevented replication of the pCP20 plasmid. The single deletion mutants, containing no antibiotic resistance cassettes could then

be used as recipient cells for further P22 transduction to produce double mutants, with resistance to one antibiotic, for example *ΔnarGHJIΔnarZYWV* Km^R. This double mutant was then transduced with the P22 lysate for *ΔnapDA* Ct^R to produce the *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant, resistant to both kanamycin and chloramphenicol. Two successive purifications on green plates, and PCR amplification confirmed successful transduction of the mutations.

2.6 Growth Curves

2.6.1 Aerobic

Single colonies of wild-type SL1344, and SL1344 mutant strains were picked from freshly streaked LB agar plates and cultured in 5ml LB, M9, or MGM broth at pH7, overnight at 37℃ in aerated conditions (250r pm). The SL1344 strain of *Salmonella* is a histidine auxotroph, due to a *his*G point mutation, so immediately prior to use, 0.02% of filter-sterilized histidine was added to the M9 minimal medium. Subsequently, 1000-fold dilutions of these overnight cultures were cultivated in triplicates in 25ml final volume of the appropriate pre-warmed media in 250ml flasks, in aerated conditions at 250rpm. In order to establish colony forming units from these assays, samples were taken of each culture, 10-fold serially diluted in Phosphate Buffered Saline (PBS), and plated on LB agar in order to determine the number of viable bacterial cells. This was repeated three times, and provided the data required to calculate viability from the optical density measurements in later assays. Additionally, 1ml of each culture was taken immediately following inoculation, for measurement of the optical density (OD) using a spectrophotometer set to take measurements at 600nm. Subsequent repeats of this procedure were carried out one hour after inoculation, then at 30 minute intervals for five hours. Colony forming units

(CFUs) were counted for each time point. A growth curve showing the CFUs/ml of culture was created, and association with the OD reading was established.

2.6.2 Acid shock assays

Single colonies of wild-type SL1344 and SL1344 mutant strains were picked from LB agar plates and cultured in 5ml LB pH7 overnight at 37℃ in aerated conditions (250rpm). 1000-fold dilutions of these overnight cultures were subsequently cultivated in triplicate in 25ml final volume of pre-warmed LB media in 250ml flasks, in aerated conditions. After 2 hours (early log phase), 4 hours (mid log phase) and 7 hours (late log phase), the subculture was 25-fold diluted in pre-warmed LB pH7 and pH3. Samples were taken at 5, 15, 30 and 45 minute intervals from each 1:25 subculture, serially diluted in Phosphate Buffered Saline (PBS), and plated on LB agar in order to determine the number of viable bacterial cells. Colony forming units (CFUs) were counted for each time point.

2.6.3 Artificial gastric juice assay

Artificial gastric juice was prepared in accordance with the methods described by Mandalari *et al* (Mandalari, Faulks et al. 2008).

Single colonies of wild-type SL1344, and SL1344 mutant strains were picked from LB agar plates and cultured in 5ml LB pH7 overnight at 37℃ in aerated conditions (250rpm). 1000-fold dilutions of these overnight cultures were subsequently cultivated in triplicate in 25ml final volume of pre-warmed LB media in 250ml flasks, in aerated conditions. After 4 hours (mid log phase), 5.5 hours (mid/late log phase) and 8 hours (late/stationary log phase), subcultures were diluted 25-fold in pre-warmed gastric juice, with and without additional enzymes

(pepsin and gastric lipase analogue). Samples were taken at 15, 30 and 45 minute intervals from each 1:25 subculture, serially 10-fold diluted in Phosphate Buffered Saline (PBS), and plated on LB agar in order to determine the number of viable bacterial cells. Colony forming units (CFUs) were counted for each time point.

2.6.4 Acid / Nitrite Assay

Single colonies of wild-type SL1344, and SL1344 mutant strains were picked from LB agar plates and cultured in 5ml LB pH7 overnight at 37℃ in aerated conditions (250rpm). 1000-fold dilutions of these overnight cultures were subsequently cultivated in triplicate in 25ml final volume of pre-warmed LB media in 250ml flasks, in aerated conditions. After 2 hours (early log phase), 4 hours (mid log phase) and 7 hours (late log phase), 25-fold diluted subculture in pre-warmed LB pH7 and pH3. Sodium Nitrate ($NaNO_2$) was added to 761.5μM final concentration (McKnight, Smith et al. 1997). Samples were taken at 5, 15, 30 and 45 minute intervals from each 1:25 subculture, 10-fold serially diluted in Phosphate Buffered Saline (PBS), and plated on LB agar in order to determine the number of viable bacterial cells. Colony forming units (CFUs) were counted for each time point.

2.6.5 Anaerobic

Single colonies of wild-type SL1344, and SL1344 mutant strains were picked from LB agar plates and cultured in LB or MGM or GN broth, overnight at 37℃ in static conditions. The bottles were filled to the top and sealed with a screw cap and parafilm was applied so that the bottles became anaerobic quickly. 10-fold dilutions (250μl in 2.5ml medium) of these overnight cultures were subsequently inoculated into glass cuvettes, sealed with rubber septa and sparged with

nitrogen gas for 5 minutes. The cuvettes were cultured anaerobically at 37℃ without shaking. Growth was monitored by measuring the absorbance OD at 600nm (ΔOD_{600nm}) every hour. Before every reading was taken, the cultures were mixed by inversion to prevent settling of the bacterial cultures.

2.6.6 Preparation of NO solution

To ensure that nitrite would not form, water adjusted to pH3 was prepared by addition of HCl. In a 5ml glass bottle, sealed with a rubber septum and parafilm, 3ml of pH3 water was sparged with nitrogen gas to remove all oxygen present. A gas-tight Hamilton syringe was used to add 7ml of nitric oxide gas in 1ml stages, by bubbling the gas through the liquid. The NO gas had been scrubbed with 0.1M NaOH to remove impurities. This method produced a saturated NO solution of approximately 2mM concentration, and was used immediately following preparation to prevent degradation of the solution.

2.6.7 Sensitivity of *Salmonella* strains to NO

Cultures for anaerobic growth curves were prepared as outlined above. As the ΔOD_{600nm} absorbance reached 0.1, NO solution was added to a final concentration of 40μM in the cuvettes, using a gas-tight Hamilton syringe. The concentration of 40μM NO is physiologically relevant and equates to the NO concentration which is expected to be encountered in the macrophages of the mammalian innate immune response to infection. Following NO addition, monitoring of growth during incubation at 37℃ was con tinued by measurement of ΔOD_{600nm}.

2.7 Motility assays

Minimal medium agar plates, with 10g bacto-tryptone, 5g NaCl and 4g agar (0.4%) per litre were made. These were inoculated with 1µl of overnight cultures of the strains to be tested, and 1:10 dilutions of the overnight cultures were used to assess the ΔOD_{600nm}. The ΔOD_{600nm} measurement was then used to calculate the number of bacterial cells in the overnight culture using an Excel spreadsheet for inoculum calculations based on an $\Delta OD_{600nm} \approx 1.0 = 1.7x10^9$ viable cells (G.Rowley). This allowed adjustments to be made when comparing motility between strains, depending on bacterial numbers in the original inoculum. The diameters of the motility zones on the plates were measured in millimetres.

2.8 Epithelial cells – invasion assays

Epithelial cells of the immortalised epithelial HeLa cell line were cultured in DMEM medium (Sigma) with 10% foetal bovine serum, 1% L-Glutamine and 1% non-essential amino acids, at 37℃ and 5% CO_2.

S. Typhimurium strains were grown on LB plates, and colonies were picked and suspended in 3ml PBS to form a cloudy suspension. The OD_{600nm} of a 1:10 dilution of the suspension was taken so that bacterial numbers present could be calculated. Dilution ratio to ensure a multiplicity of infection (MOI) of 1:1 was calculated.

If induction of the SPI-1 apparatus was required, *S*. Typhimurium strains were grown overnight in LB, and a 1:10 subculture in LBS cultured for one hour and 45 minutes, to activate the SPI-1 T3SS in the bacteria. The bacteria were again incubated with epithelial cells at a multiplicity of infection ratio of 1:1. Bacteria which remained extracellular were killed after 30 minutes by the addition of 30µg/ml gentamycin. After 30 minutes, the killing medium was replaced with a maintenance medium containing only 5µg/ml gentamycin. After 1 hour or 5

hours, the medium was removed, and the monolayer of epithelial cells was washed twice with sterile PBS. The epithelial cells were lysed using a solution of 0.1% SDS, and serial dilutions of the intracellular bacteria were plated on selective plates and counted after overnight incubation at 37℃.

Epithelial cells of the immortalised epithelial HeLa cell line were cultured in DMEM medium (Sigma) with 10% foetal bovine serum, 1% L-Glutamine and 1% non-essential amino acids, at 37℃ and 5% CO_2.

2.9 Macrophage cells – gentamycin protection assays

Macrophage cells of the murine cell line RAW264.7 were cultured in MEM medium (Sigma) supplemented with 10% foetal bovine serum, 1% L-Glutamine and 1% non-essential amino acids, at 37℃ and 5% CO_2.

S. Typhimurium strains were grown on LB plates, and colonies were picked and suspended in 3ml PBS to form a cloudy suspension. The OD_{600nm} of a 1:10 dilution of the suspension was taken so that bacterial numbers present could be calculated. Dilution ratio to ensure a multiplicity of infection (MOI) of 1:1 was calculated, and the bacteria were incubated with macrophage cells at a MOI of 1:1. Bacteria which remained extracellular were killed after 60 minutes by the addition of 50μg/ml gentamycin. After 30 minutes, the medium was replaced with one containing only 10μg/ml. After 30 minutes for the 2 hour timepoint, or 22.5 hours for the 24 hour timepoint, this maintenance medium was removed, and the monolayer of macrophage cells was washed three times with sterile PBS. The epithelial cells were lysed using a solution of 0.1% SDS and 1% Triton-X, and serial dilutions of the intracellular bacteria were plated on selective plates and counted after overnight incubation at 37℃.

2.10 Murine infection virulence assays

Infections were carried out in accordance with the requirements of the Home Office, under the Scientific Procedures (Animals) Act 1986.

2.10.1 Oral infection

As the infection progresses in mice, bacteraemia and development of fever causes progressive changes in the behaviour and appearance of the animals, giving identifiable symptoms. Four sets of symptoms (reduced activity, anorexia, hunched posture, ruffled fur) were scored, depending on the severity of each symptom observed, in each mouse at least twice daily, to ensure that unnecessary suffering was avoided. Mice were sacrificed at the latest on day five following infection, and gut contents were isolated from stomach, proximal/mid/distal small intestine, caecum and colon, weighed and homogenised in 200µl PBS. Tissue samples were also collected from small intestine Peyer's Patches, spleen and liver. Peyer's Patches were homogenised in 200µl PBS, and liver and spleen in 5ml PBS. All content and Peyer's Patch tissue samples were serially diluted and plated on XLD agar containing streptomycin to exclude commensal enterobacteria. Spleen and liver tissue samples were plated on LB agar. Plates were incubated overnight at 37℃. Viable colonies were then counted, and CFU/g content/tissue calculated.

2.10.1.1 SPI-1 induction method

Salmonella strains were cultivated overnight in LB broth at 37℃ in aerated conditions (250rpm). A 10-fold dilution of each strain was made in pre-warmed LBS, and incubated at 37℃ for 1 hour and 45 minutes. Cells were harvested by centrifugation at room temperature at 8000rpm for 10 minutes. The supernatant

was removed and cells were washed twice in PBS. The cells were subsequently concentrated so that $5x10^8$ bacterial cells were contained in a 20µl dose volume.

BALB/c mice were starved for 16 hours prior to infection. Water was removed from cages up to four hours prior to infection. Animals were infected orally with $5x10^8$ bacterial cells of each strain tested. Inoculae were serially diluted in PBS and plated on XLD containing streptomycin.

2.10.1.2 Without SPI-1 induction

Salmonella strains were cultivated overnight in 45ml LB broth at 37℃ in static conditions. Cells were harvested by centrifugation at 4℃ room temperature at 4000rpm for 15 minutes. The supernatant was removed and cells were resuspended in 3ml PBS. The ΔOD_{600nm} measurement was then used to calculate the number of bacterial cells in the inoculum using an Excel spreadsheet for inoculum calculations based on an $\Delta OD_{600nm} \approx 1.0 = 1.7x10^9$ viable cells (G.Rowley).The inoculum was subsequently diluted in PBS so that $5x10^7$ bacterial cells were contained in a 20µl dose volume.

BALB/c mice were starved for 16 hours prior to infection. Water was removed from cages up to four hours prior to infection. Animals were infected orally with $5x10^7$ bacterial cells of each strain tested. Inoculae were serially diluted in PBS and plated on XLD containing streptomycin.

2.10.2 Intra-peritoneal competitive index infection

Salmonella strains were cultivated overnight in 45ml LB broth at 37℃ in static conditions. Cells were harvested by centrifugation at 4°C, 4000rpm for 15 minutes. The cells were resuspended in 3ml PBS, and the ΔOD_{600nm} of a 1:10 dilution used to calculate the dilution required to ensure that $2x10^3$ cells, a 50/50 mix of wild type and the mutant strain, would be administered in a 200µl dose.

Animals were infected by injection into the intra-peritoneal cavity with a mixed inoculum of $2x10^3$ bacterial cells. The inoculae were serially diluted in PBS and plated on LB with streptomycin, and LB with streptomycin and kanamycin to select for the mutant strain only. Once the numbers of bacteria recovered had been calculated, the competitive index (CI) value was established by dividing the output ratio of both the strains by the input ratio of the two strains.

The four sets of symptoms (reduced activity, anorexia, hunched posture, ruffled fur) were again scored, depending on the severity of each symptom observed, in each mouse at least twice daily, to avoid unnecessary suffering of the animals. Mice were sacrificed at the latest on day five following infection and the liver and spleen removed, weighed and homogenized in 5ml PBS. Spleen and liver tissue samples were plated on LB agar. Plates were incubated overnight at 37℃. Viable colonies were then counted, and CFU/g content/tissue calculated. The competitive index calculation (CI value) was calculated for each strain. This is done by dividing the CFU for each strain by the total CFU of all the strains to obtain a ratio of each strain in each organ, and related back to the original inoculum ratios for the strains. A value of around one showed that a mutant strain was capable of competing (and infecting) just as well as the wild type strain. Conversely, very low CI values of significantly less than one would demonstrate that a mutant strain was attenuated for infection in comparison to the wild type strain.

2.11 β-Galactosidase Activity Assays

2.11.1 Aerobic Assays

Aerobic β-Galactosidase Assays were carried out on strains of *S*. Typhimurium which had been transformed with the low-copy number *lacZ* promoter fusion reporter plasmid pMP220.

10ml cultures in LB with tetracycline to select for the plasmid were grown overnight, and diluted 1:10 in fresh medium and grown to mid-log, demonstrated by OD_{600nm} of close to 0.6. 1ml aliquots in micro-centrifuge tubes were incubated on ice for twenty minutes to stop further growth, then the cells were pelleted by centrifugation at 6000rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended to a volume of 1ml in chilled Z-buffer (see Section 2.11.2 below). The OD_{600nm} was recorded.

Diluted 0.5ml cells in Z-buffer in further 0.5ml Z-buffer. Added 100µl chloroform and 50µl 0.1% sodium dodecyl sulphate (SDS), vortexed to mix and equilibrated at 28℃ for 5 minutes.

Start the β-galactosidase activity reaction by adding 0.2ml ONPG solution (see Section 2.11.3 below), which acts as a colour substrate to the enzyme, and a pale yellow colour similar to LB broth developed when incubated at 28℃. The reaction was stopped by adding 0.5ml 1M Na_2CO_3, which stops the reaction by raising the pH to 11. The time taken for the colour to develop was accurately recorded.

A 1ml aliquot of the reaction mixture was centrifuged for 5 minutes at 13000rpm to remove debris and chloroform, and the OD_{550nm} and OD_{420nm} recorded.

The units of enzyme activity were calculated in Miller Units as follows:

$$\frac{1000 \times (OD_{420} - (1.75 \times OD_{550}))}{\text{time (minutes)} \times \text{volume (mls)} \times OD_{600}}$$

2.11.2 Z-Buffer

Dissolve in H_2O, 0.80g $Na_2HPO_4.7H_2O$ (0.06M); 0.28g $NaH_2PO_4.H_2O$ (0.04M); 0.5ml 1M KCl (0.01M); 0.05ml 1M $MgSO_4$ (0.001M); 0.135ml β-mercaptoethanol (BME) (0.05M); pH adjusted to 7.0. Make up to 50ml with H_2O.

2.11.3 O-nitrophenol-β-D-galactopyranoside (ONPG)

ONPG to a final dilution of 4mg/ml dissolved in phosphate buffer (0.80g $Na_2HPO_4.7H_2O$ (0.06M); 0.28g $NaH_2PO_4.H_2O$ (0.04M); dissolved in H_2O, adjusted to pH 7.0).Make up to 50ml with H_2O.

2.11.4 Anaerobic Assays

Anaerobic β-Galactosidase Assays were also carried out on strains of *S*. Typhimurium transformed with the low-copy number *lacZ* promoter fusion reporter plasmid pMP220.

Strains were cultured in universals filled with LB with tetracycline to select for the plasmid, overnight at 37℃ in static conditions. The bot tles were filled to the top and sealed with a screw cap and parafilm was applied so that the bottles became anaerobic quickly. The overnight cultures were diluted 1:10 in Glycerol Nitrate (GN) medium in 3ml optical glass cuvettes, which were sealed with rubber septa and sparged with nitrogen gas for 5 minutes. The cuvettes were incubated at 37℃ for six hours. 1ml aliquots in micro-centrifuge tub es were incubated on ice for twenty minutes to stop further growth, then the cells were pelleted by centrifugation at 6000rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended to a volume of 1ml in chilled Z-buffer. The OD_{600nm} was recorded.

Added 100µl chloroform and 50µl 0.1% SDS, vortexed to mix and equilibrated at 28℃ for 5 minutes.

The β-galactosidase activity reaction was started by adding 0.2ml ONPG solution, and incubated at 28℃. The reaction was stopp ed by adding 0.5ml 1M Na_2CO_3. The time taken for the colour to develop was accurately recorded.

A 1ml aliquot of the reaction mixture was centrifuged for 5 minutes at 13000rpm to remove debris and chloroform, and the OD_{550nm} and OD_{420nm} recorded.

The units of enzyme activity were calculated in Miller Units as shown above.

Chapter 3 –NorV, NrfA and HmpA, analysis of the activity of theSL1344Δ*norV*Δ*nrfA*Δ*hmpA* triple mutant in the lumen of the murine gastrointestinal tract

3.1 Introduction

Work carried out previously has investigated the sensitivity of the *SalmonellaSL1344ΔnorVΔnrfAΔhmpA*triple deletion mutant to exposure to nitric oxide using *in vitro* methods (Mills, Rowley et al. 2008). This suggested an additive effect of NorV and NrfA in the defence against NO, and also a minor role for HmpA in the anaerobic detoxification of NO, with a larger role for aerobic NO detoxification by HmpA. Although the genes required for detoxification of nitric oxide *in vitro* may be established by using *in vitro* methods, in order to ascertain the effect on virulence of deletion of genes likely to be required for nitric oxide detoxification *in vivo*, the best model to use is the murine model. Only in this way can any physiological role be determined for any particular gene, or combination of genes.

In this regard, other previous work has shown that the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant was not attenuated after intraperitoneal infection of mice, or in cultured murine macrophages (G. Rowley, unpublished data). However, intraperitoneal infection of mice does not expose *Salmonella* to the harsh environment of the murine gastrointestinal tract, containing various antimicrobial compounds together with the anaerobiosis generally observed in the gastrointestinal tract. The work carried out under the auspices of this project, using oral infection of mice, ensured that the mutant would be challenged within the gut environment via a more 'natural' route of infection in a truly *in vivo* fashion. Hence, work on this project aimed to complete the characterisation of the *SL1344 ΔnorVΔnrfAΔhmpA* triple deletion mutant (strain called JH3518).

The flavorubredoxin NorV of *E. coli* is a NO reductase which, sensitive to oxygen, assists NO detoxification in *E. coli* in anaerobiosis (Gardner, Helmick et al. 2002; Gomes, Giuffre et al. 2002). NorV is required by *E. coli* in the presence of the NO^+ donor sodium nitroprusside (SNP) (Hutchings, Mandhana et al. 2002),

but since this chemical source of NO also produces other toxic substances, such as cyanide, there may in this case be other forces at work apart from NO sensitivity (Mills, Rowley et al. 2008). Upregulation of *norV* expression has been reported under conditions where NO has been increased from various sources (Flatley, Barrett et al. 2005; Justino, Vicente et al. 2005; Pullan, Gidley et al. 2007). NorR is the NorV regulator which responds to the presence of NO to cause this phenomenon (Hutchings, Mandhana et al. 2002; Mukhopadhyay, Zheng et al. 2004). Within macrophages, in response to the NO burst, norV expression is upregulated (Eriksson, Lucchini et al. 2003), though *E. coli* survives here even without the NorV enzyme (Pullan, Gidley et al. 2007).

The cytochrome *c* nitrite reductase NrfA has been structurally and spectropotentiometrically characterised (Bamford, Angove et al. 2002; Burlat, Gwyer et al. 2005; Clarke, Kemp et al. 2008). It catalyses reduction of nitrite to ammonia, and also reduces NO (Poock, Leach et al. 2002; van Wonderen, Burlat et al. 2008). Poock *et al* also showed that in *E. coli*, a *nrfA* mutant was more sensitive to NO in anaerobic conditions (Poock, Leach et al. 2002). The NO sensitive repressor NsrR is responsible for regulation of NrfA expression in *E. coli* (Filenko, Spiro et al. 2007). Recent work has also shown that in *E. coli*, NsrR downregulates activation of the nrf promoter which is in turn FNR-dependent (Browning, Lee et al. 2010)

It has previously been established that HmpA, the cytoplasmic flavohaemoglobin enzyme in *Salmonella* and *E. coli*, works as a NO dioxygenase (Crawford and Goldberg 1998; Gardner, Gardner et al. 1998; Hausladen, Gow et al. 1998). The activity of this enzyme as an anaerobic NO reductase required further elucidation. It is known to reduce NO to nitrous oxide in the absence of oxygen in *E. coli* (Kim, Orii et al. 1999), and in *Salmonella*, anaerobic sensitivity to S-nitrosoglutathione (GSNO) has been demonstrated (Crawford and Goldberg 1998). Some work had suggested that HmpA has a role in survival and

replication of *Salmonella* in macrophages (Stevanin, Poole et al. 2002; Bang, Liu et al. 2006; Gilberthorpe, Lee et al. 2007) This may also offer some explanation of observations that virulence of *Salmonella* in mice is also supported by HmpA (Bang, Liu et al. 2006).It has been shown that *Salmonella* has the ability to produce NO endogenously, and it is likely that HmpA consumes this NO, consequently preventing inactivation of the FNR by the reaction of NsrR to the NO, thus maintaining NO homeostasis by preventing transcription of the FNR regulon which incorporates NrfA (Gilberthorpe and Poole 2008). NsrR is a key regulator in the circumvention of nitrosative stress for *S*. Typhimurium (Gilberthorpe, Lee et al. 2007), acting as it does as a transcriptional repressor (Tucker, Hicks et al. 2008).

The infection in mice which is caused by *S*. Typhimurium is symptomatically very similar to that which is caused by *S*. Typhi in humans. As early as 1974, Carter and Collins were able to demonstrate that following infection from three points of inoculation, the site of bacterial penetration was the distal ileum (Carter and Collins 1974). The bacterial inoculum can be administered either orally or intraperitoneally. Earlier work had already shown that following intra-peritoneal infection of BALB/c mice and murine macrophages, the triple *SL1344 ΔnorVΔnrfAΔhmpA* mutant was not attenuated *in vivo* (Rowley et al., unpublished work). Since intra-peritoneal inoculation, in which the inoculum is dispensed directly into the peritoneal cavity which houses the intestines, stomach and liver, effectively bypasses the normal route of ingestion for enteric pathogens, it was decided for this work to use oral infection, which would more closely mimic the natural route of infection. Indeed, some work has shown that bacterial cells may be killed by low pH, such as would be encountered in the acidic stomach, combined with the presence of nitrate (Dykhuizen, Frazer et al. 1996). Although the use of cultured cells, such as macrophages, may provide useful complementary data, since macrophages do provide an important niche

for replication of *Salmonella* during the infection process, it cannot be considered definitive, since it is not possible to replicate exact *in vivo* conditions during their use, so such models effectively provide *ex vivo* information (Mills, Rowley et al. 2008).

3.1.1 Attenuation of the *SL1344ΔnorVΔnrfAΔhmpA* triple mutant in the lumen of the murine gastrointestinal tract

To determine whether the triple mutant was attenuated following oral infection, bacterial loads in a range of organs was calculated post infection. The first experiment which was conducted to provide a comparison between population levels reached *in vivo* by both the wild type and *ΔnorVΔnrfAΔhmpA* triple mutant appeared to show that some attenuation occurred (Figure 7). The inoculum was prepared using the SPI-1 inducing method described earlier. Bacterial loads from both the contents of various parts of the gastrointestinal tract and tissue samples are based on the numbers of viable CFUs which were extracted. Both strains were present in all intestinal compartments. Strikingly, the wild type strain reached higher population levels in all samples tested, particularly in the upper small intestine, than the mutant, suggesting that the *norV*, *nrfA* and *hmpA* genes might play an important role in the early stages of *Salmonella* infection when *Salmonella* resides in the gastrointestinal tract lumen. The mutant also reached lower levels than its parental strain in the spleen and liver. This last result was different to previous results obtained by Rowley *et al*. that showed that intraperitoneal infection of mice resulted in similar colonisation of the spleen and liver by both the triple mutant and the wild type strain. However, we speculated that the difference observed between the two strains here could result from the attenuation observed in the intestine of the animals. Less mutant cells might be able to survive and/or replicate inside the gastrointestinal tract lumen, so less

bacterial cells might maintain contact with and invade the epithelium, and hence reach the spleen and liver. We noted a high variability in the SL1344 wild type viable counts in the upper intestinal compartments, such as stomach and duodenum. This can result from the coprophagic habits characteristic of rodents; one animal might ingest some faecal pellets containing high levels of *Salmonella* when other animals from the same group might not, or not at the same time. This could bias the average population levels calculated in the upper intestinal compartments. Nonetheless, this initial set of experiments suggested a potential role of NorV, NrfA and HmpA in the upper part of the gastrointestinal tract, such as the stomach.

However, subsequent repeat experiments failed to reproduce the results which were initially observed (Figure 8). It can be seen that any difference between the wild type and mutant survival in the gastrointestinal tract had been eroded, and systemically also, there is nothing to choose between the strains in terms of viable counts of bacteria which were harvested from the tissue samples.

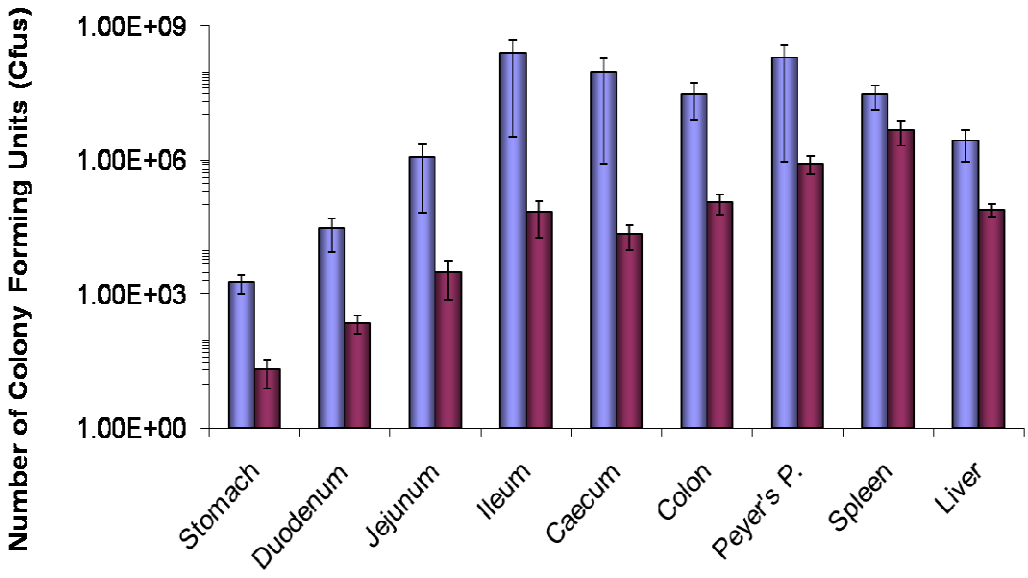


Figure 7 - Comparison of population levels of SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant in murine gut contents and tissue. Two groups of BALB/c mice (13 mice in total) were infected with separate strains of interest. Five days following infection, the mice were sacrificed and gut contents and tissue were harvested. The SL1344 Wild Type (blue bars) was present in higher numbers than the SL1344 *ΔnorVΔnrfAΔhmpA* triple mutant (mauve bars). Error bars show the Standard Error of the Mean.

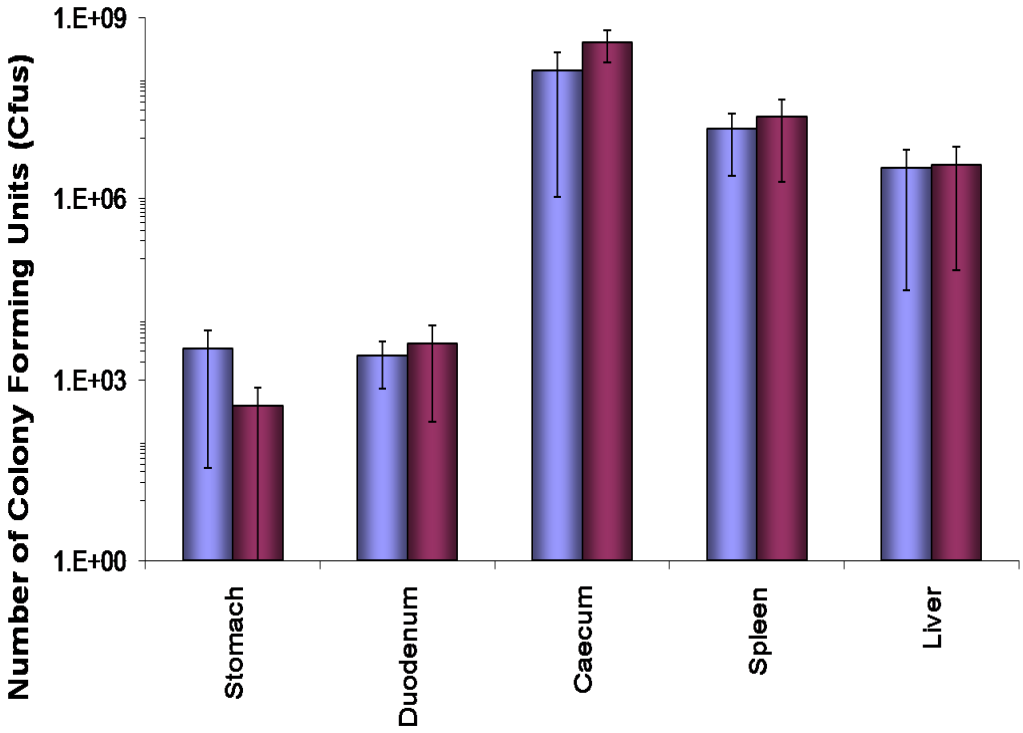


Figure 8 - Later comparison of population levels of SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant in murine gut contents and tissue. Two groups of BALB/c mice (10 mice in total) were infected with separate strains of interest. Five days following infection, the mice were sacrificed and gut contents and tissue were harvested. There was no difference in bacterial numbers present between SL1344 Wild Type (blue bars) and the SL1344 *ΔnorVΔnrfAΔhmpA* triple mutant (mauve bars). Error bars show the Standard Error of the Mean.

3.1.2 *In vitro* assays to test the SL1344*ΔnorVΔnrfAΔhmpA* triple mutant

3.1.2.1 Acid shock assays

Growth curves carried out previously had shown that there was no difference in growth rate between SL1344 wild type and the SL1344 *ΔnorVΔnrfAΔhmpA* triple mutant in either LB or M9 minimal media in aerobic conditions. However, initially at least, the oral infection of BALB/c mice described in Section 3.1.1 suggested that there was attenuation of the SL1344 *ΔnorVΔnrfAΔhmpA* triple mutant when administered via the oral route. Since *Salmonella* is challenged by exposure to conditions of very low pH during gastric transit, it was decided that acid shock assays would also be performed. Conditions in the mammalian stomach are also at least partially aerobic (Rath, Wilson et al. 1999; Xu, Xu et al. 2001).

The acid shock assays were carried out aerobically at neutral (pH7) and acidic (pH3) in LB medium, and showed that, regardless of the log phase to which the cultures had been grown, there is little difference between SL1344 wild type and the triple mutant (Figures 9, 10, 11) in their response to a highly acidic environment.

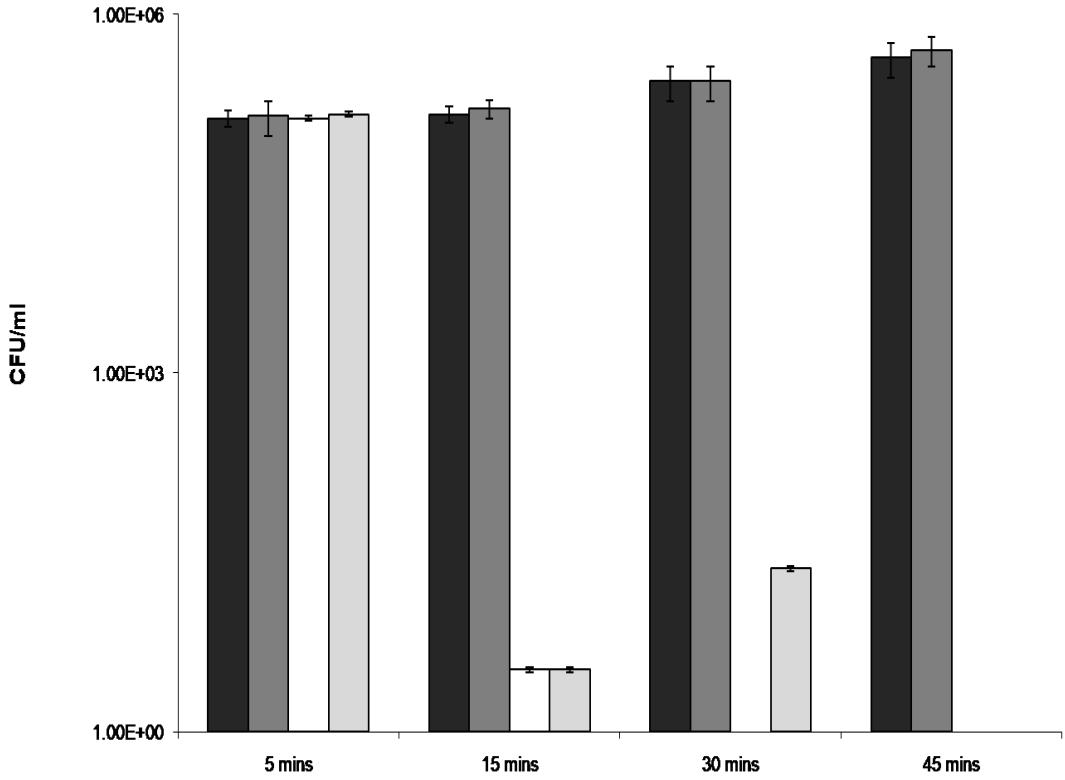


Figure 9 - Early log phase acid shock assay for *Salmonella* SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant. Survival of early log phase *Salmonella* strains was assayed in Luria-Bertani broth at both pH7 and pH3 (by the addition of HCl). The data are the averages from three independent experiments, and the error bars show standard error of the mean. There was no difference in the growth at pH7 between the two strains, Wild Type, pH7 (black bars) and Mutant, pH7 (dark grey bars). Both strains in pH3 broth were seriously debilitated within 15 minutes of their first exposure, Wild Type pH3 (open bars) and Mutant pH3 (pale grey bars). By 45 minutes following exposure, no viable bacteria survived, and this was the same for both the SL1344 wild type and the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant.

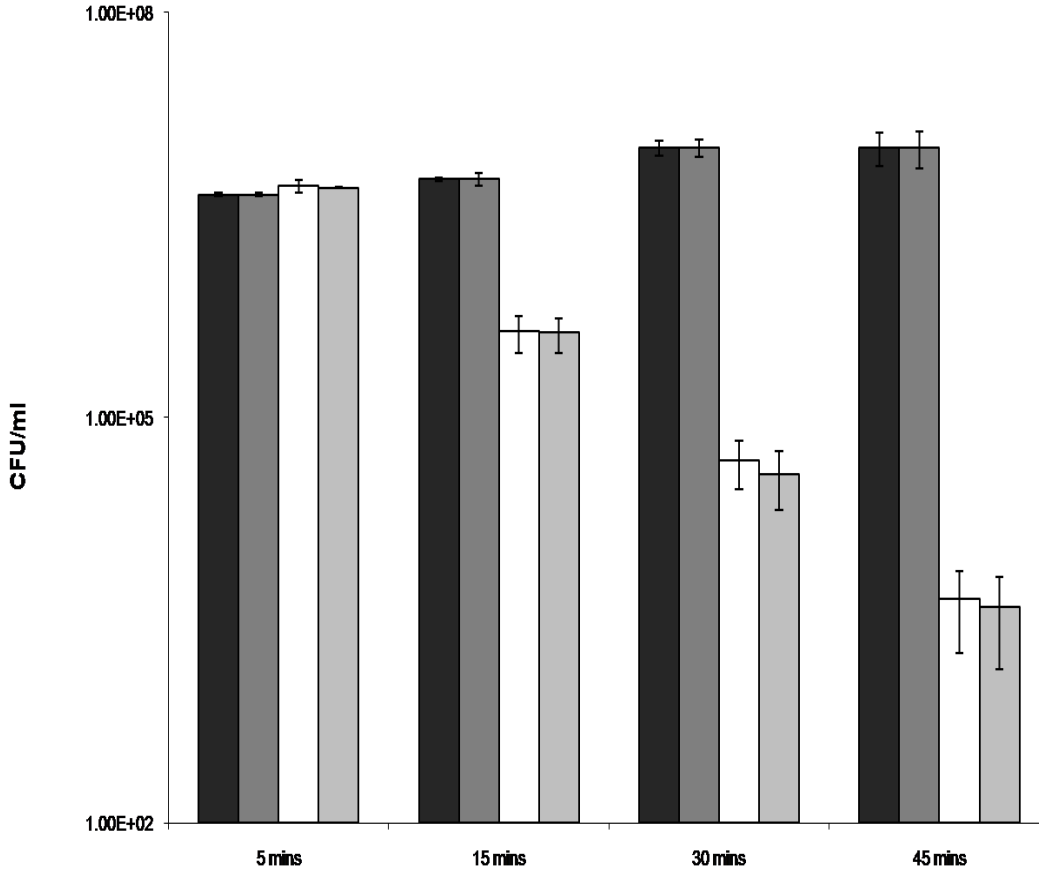


Figure 10 - Mid log phase acid shock assay for *Salmonella* SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant. Survival of mid log phase *Salmonella* strains was assayed in Luria-Bertani broth at both pH7 and pH3 (by the addition of HCl). The data are the averages from three independent experiments, and the error bars show standard error of the mean. There was no difference in the growth at pH7 between the two strains, Wild Type, pH7 (black bars) and Mutant, pH7 (dark grey bars). Both strains in pH3 broth were affected within 15 minutes of their first exposure, Wild Type pH3 (open bars) and Mutant pH3 (pale grey bars). The number of viable bacteria which survive reduced continuously over time up to 45 minutes following exposure and there was no difference in the response to pH3 between the SL1344 wild type and the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant.

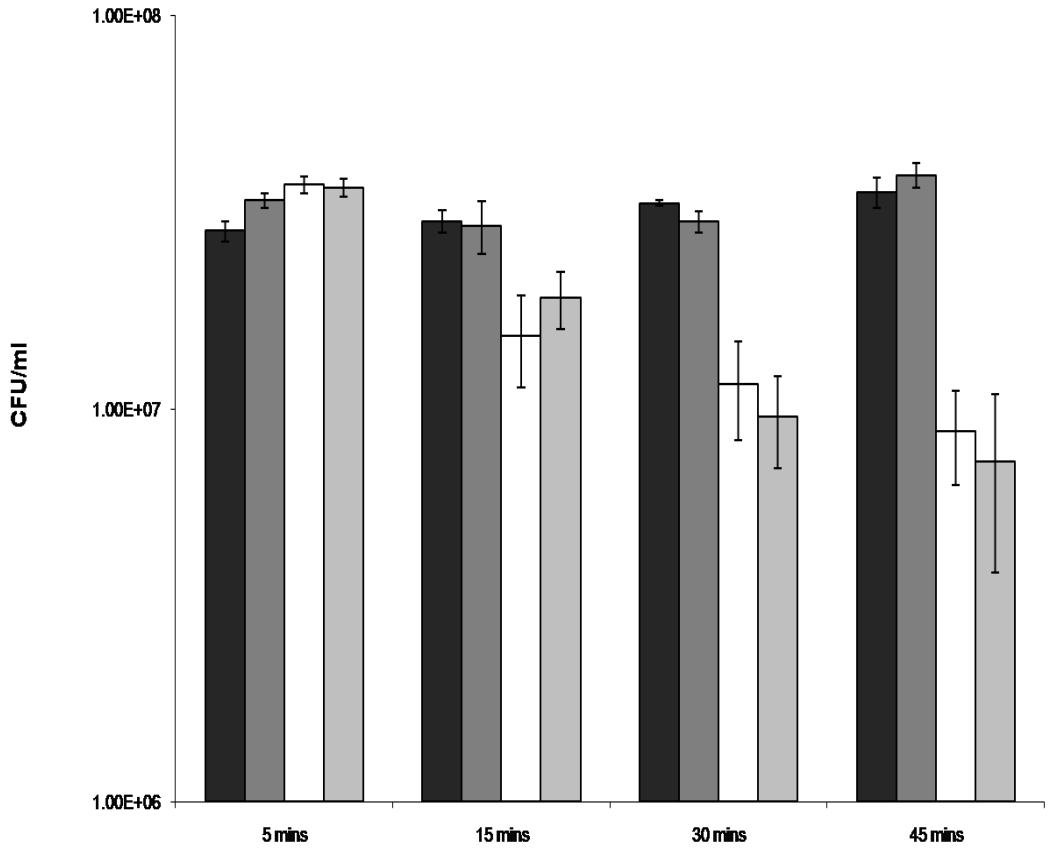


Figure 11 - Late log phase acid shock assay for *Salmonella* SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant. Survival of late log phase *Salmonella* strains was assayed in Luria-Bertani broth at both pH7 and pH3 (by the addition of HCl). The data are the averages from three independent experiments, and the error bars show standard error of the mean. There was no difference in the growth at pH7 between the two strains, Wild Type, pH7 (black bars) and Mutant, pH7 (dark grey bars). Both strains in pH3 broth were affected within 15 minutes of their first exposure, Wild Type pH3 (open bars) and Mutant pH3 (pale grey bars). The number of viable bacteria which survive reduced continuously over time up to 45 minutes following exposure and there was no difference in the response to pH3 between the SL1344 wild type and the *SL1344 ΔnorVΔnrfAΔhmpA* triple deletion mutant.

In early-log phase, both the SL1344 wild type and mutant were susceptible to acid shock by the time they had been exposed to pH3 conditions for 15 minutes, and no viable bacteria remained in the pH3 cultures (Figure 9). This result is analogous to those achieved in other work, particularly where no pre-conditioning to induce the acid tolerance response by exposing to a lower pH has been carried out first (Foster 1991).

In mid-log phase, once again there was a reduction in viable bacterial numbers noticeable by 15 minutes following exposure to pH3, with the numbers reducing progressively over time (Figure 10). This effect was also apparent for late-log phase cultures, although to a lesser degree, showing that late-log bacteria had a higher resistance to the effects of acid shock than those in earlier growth phases (Figure 11). Overall, however, there was no discernible difference between SL1344 wild type and the *SL1344 ΔnorVΔnrfAΔhmpA* triple mutant under these acid shock conditions.

3.1.2.2 Artificial gastric juice assay

Work by Mandalari *et al* at the Institute of Food Research, Norwich (IFR) has resulted in the development of an artificial gastric juice, which can be used in a model gastric system to simulate digestion of food items (Mandalari, Faulks et al. 2008). The artificial gastric juice contains egg L-α-phosphatidylcholine, used to construct phospholipid vesicles within the juice, together with gastric mucosal enzymes pepsin and trypsin. The gastric juice was adjusted to pH3, to match the test carried out previously in the acid shock assay. However, the pH at which these enzymes operate *in vivo* is pH2.5. To test whether there was any difference between a purely chemical acid shock assay, and that which might be experienced in a more natural model, both SL1344 wild type and the *SL1344*

ΔnorVΔnrfAΔhmpA triple mutant were exposed to this artificial gastric juice using similar methods to those of the acid shock assays.

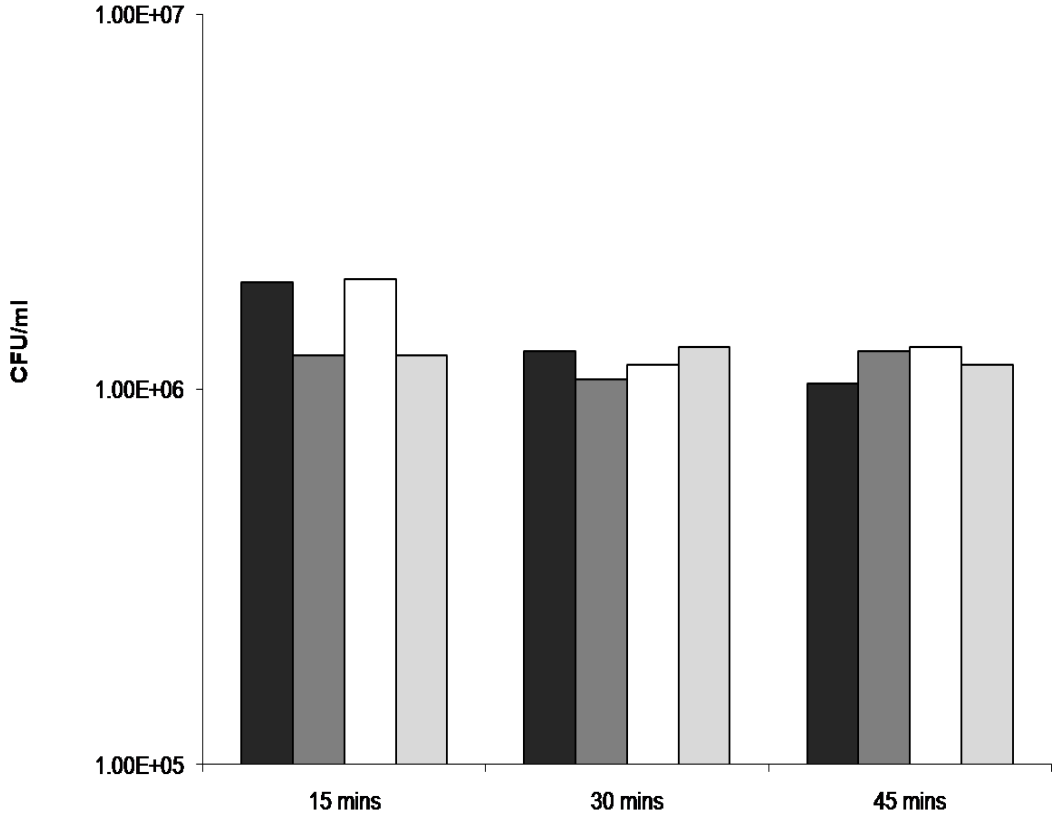


Figure 12 - Early log phase gastric fluid assay for *Salmonella* SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant. Survival of early log phase *Salmonella* strains exposed to artificial gastric juice. The data are from one experiment. There was no difference in survival the two strains, although the SL1344 Wild Type bacterial numbers (with enzymes = black bars, without enzymes = open bars) appeared to reduce between 15 and 30 minutes exposure, In contrast, numbers of the SL1344 *ΔnorVΔnrfAΔhmpA* mutant (with enzymes = dark grey bars, without enzymes = pale grey bars) remained fairly static throughout the whole period of exposure. Neither strain increased in viable bacterial numbers during the period of the assay, so growth and/or replication was inhibited in gastric fluid.

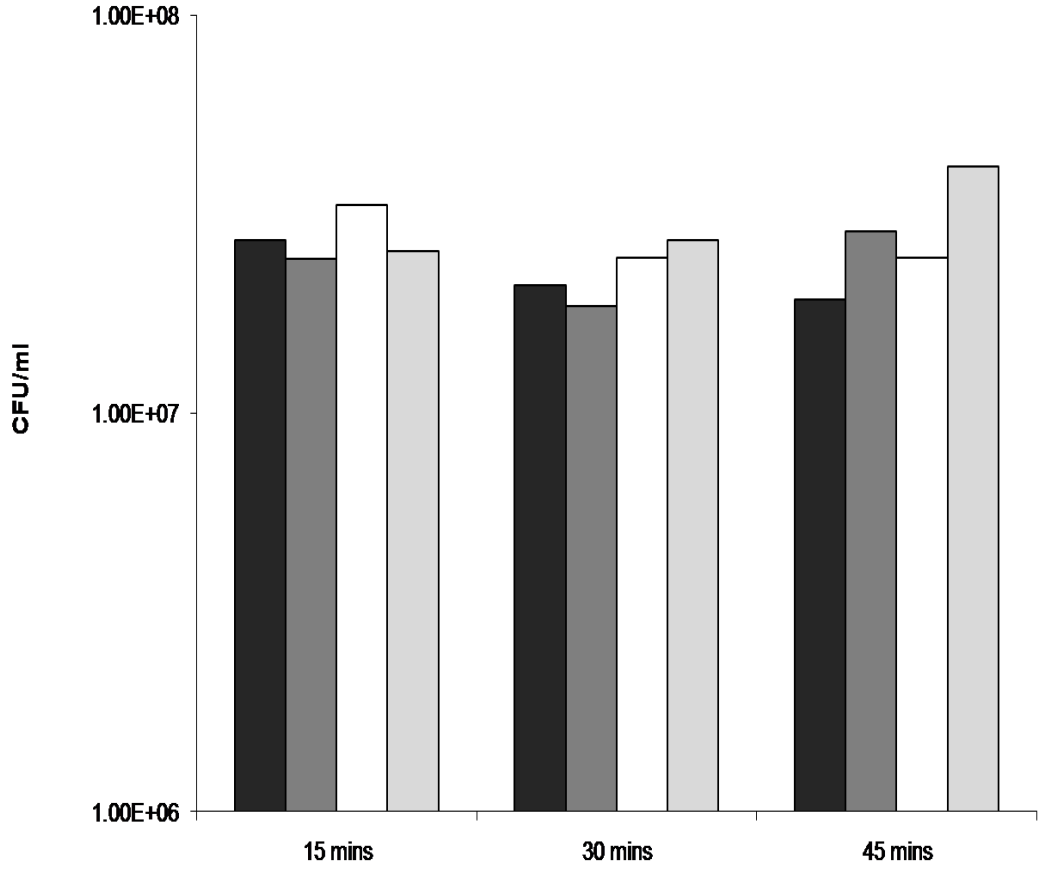


Figure 13 - Late log phase gastric fluid assay for *Salmonella* SL1344 Wild Type and SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant Survival of late log phase *Salmonella* strains exposed to artificial gastric juice. The data are from one experiment. Survival of both strains, SL1344 Wild Type bacterial numbers (with enzymes = black bars, without enzymes = open bars) and SL1344 *ΔnorVΔnrfAΔhmpA* mutant (with enzymes = dark grey bars, without enzymes = pale grey bars) was unaffected in either strain. It was interesting that, at least in the absence of pepsin and lipase enzymes, the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant may have shown some advantage over the SL1344 wild type in terms of its ability to grow

There is little discernible difference in the observed effects of the artificial gastric juice between the SL1344 wild type and the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant regardless of the growth phase of the bacteria, as shown in Figures 12 and 13. However, survival of viable colony forming bacterial units is better in the artificial gastric juice than in acidified LB for all growth phases. There did appear to be a small growth advantage to the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant over SL1344 Wild Type in the late log phase experiment, as the mutant bacterial numbers increased slightly over the period of the assay. The presence of liposomes in the gastric juice may also offer protection to the bacteria, shown by improved survival when compared to acidified LB. Since only one experiment was completed using this method, further testing would be necessary to explore both possibilities.

3.2 Discussion

Despite early results of *in vivo* experiments in which Balb/C mice were inoculated orally with both SL1344 wild type and the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant, which appeared to suggest that the mutant may have had increased virulence over the wild type, this effect could not be repeated and substantiated over subsequent experiments. The experiments were repeated several times, and this finding suggests that there are many other factors which contribute to successful *Salmonella* infections via the oral route.

Work was extended into carrying out *in vitro* investigations looking at the effects of exposure to acid, such as would occur during the passage through the mammalian stomach during a natural infection. This also demonstrated little difference in the performance between the SL1344 wild type strain compared to the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant.

Similar results were achieved following exposures of the wild type and mutant strains to synthetic gastric juice, and also an acid assay which included the addition of nitrite.

Consequently, it was concluded that under the conditions which were tested, the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant was no more or less affected than the isogenic parent.

This aligns with the findings of Rowley *et al* (unpublished data) when testing was carried out using intraperitoneal infection of Balb/C mice; their conclusions were that there was no difference in virulence between this mutant and the wild type. Such findings support the hypothesis that other mechanisms for NO detoxification exist which are induced during animal infection, and which remain to be elucidated.

It is known that *S.* Typhimurium proliferation increases during an infection if NO production by the innate immune system of mice is constrained (Umezawa,

Akaike et al. 1997; MacFarlane, Schwacha et al. 1999). The ability of mutant strains of *Salmonella* to downregulate host NO production has also been demonstrated (Eriksson, Bjorkman et al. 2000). Since there are several pathogens which, as part of their normal infection strategy, survive intracellularly within their host, it follows that there must exist ways in which they circumvent attempts by the host immune system to eradicate them whilst there. Both *S*. Typhimurium and *Mycobacterium tuberculosis*, while inducing iNOS production in macrophages, are also able to detoxify both ROS and RNI. It has been shown that *S*. Typhimurium interferes with the recruitment of components required by phagocytic host cells to mount their defensive burst (Vazquez-Torres, Xu et al. 2000).

Recent work further suggests that transporters of nitrogen species may also be crucial for governing the interplay between NO and host defences in combating the effects of *Salmonella* infection (Das, Lahiri et al. 2009). Production of NO in response to IFN-γ activation of RAW264.7 macrophages is inhibited in macrophages infected with wild-type *Salmonella*, and the nitrite transporter nirC was found to be fundamental to this effect (Das, Lahiri et al. 2009). Apparently, then, some bacterial species, including *Salmonella*, are able to exert this effect on mammalian cells and in doing so, defend themselves from attack by NO, one of the main resources available to the mammalian immune response.

Chapter 4 –Construction of nitrate reductase mutants and their characterisation *in vitro*

4.1 Introduction

A wealth of work on *E. coli*, has demonstrated its ability to use nitrate as a terminal electron acceptor during anaerobic respiration (Stewart 1982; Stewart 1988; Stewart 1993; Glaser, Danchin et al. 1995). Although *E. coli* can use oxygen, nitrate (or fumarate) as terminal electron acceptors, it will use the one with the highest redox potential which is available (Iuchi and Lin 1987). This would mean that the presence of oxygen prevents use of nitrate, the presence of which would in turn deny the use of fumarate.

Although NorV, NrfA and HmpA are implicated in the detoxification of nitric oxide, and therefore potentially in the ability of *Salmonella* to survive the presence of this reactive nitrogen intermediate during its transit through the mammalian gastrointestinal tract, it is likely that there are other participants in this process. Many virulence determinants for *Salmonella* have been identified and studied, but some aspects of *Salmonella* metabolism in relation to virulence have attracted little attention to date (Darwin and Miller 1999; Wallis and Galyov 2000; Zhang, Kingsley et al. 2003). During the course of an infection, *Salmonella* is exposed to various reactive nitrogen species. *Salmonella* Typhimurium has three distinct nitrate reductase systems, NR-A, Nap and NR-Z, as outlined above.

Also, because the gastrointestinal tract is largely anaerobic with microaerobic niches (Jones, Chowdhury et al. 2007), nitrate can be used as a terminal electron acceptor during anaerobic bacterial growth (Lundberg, Weitzberg et al. 2004). There are relatively high levels of nitrate in the mammalian gut and it is possible that bacterial infection, colonisation of the mammalian gastrointestinal tract and nitrate dissimilation are connected.

Nitrate is a source of nitrogen for many microorganisms, including *Salmonella* (Lin and Stewart 1998). In nitrate replete conditions, microorganisms employ a range of enzymes with which to assimilate the nitrogen which they require, and

for detoxification of reactive nitrogen intermediates. Nitrate reductases comprise iron-sulphur clusters and a molybdenum cofactor, while nitrite reductases, which facilitate the next stage of nitrate reduction and detoxification incorporate sirohaem with iron-sulphur clusters (Lin and Stewart 1998). However, during aerobic growth, *Salmonella* is unable to assimilate nitrate and this is also true of *E. coli* (Ingledew and Poole 1984; Glaser, Danchin et al. 1995).

Under anaerobic conditions, two systems, anaerobic respiratory nitrate reduction and nitrite reduction, facilitate the use by *Salmonella* and *E. coli* of nitrate as a sole nitrogen source under anaerobic conditions (Neidhardt 1996).

In *E. coli*, the role of the periplasmic nitrate reductase, Nap, has been shown to be the scavenging of nitrate in conditions where the availability of nitrate is limited (Potter, Millington et al. 1999). By growing a series of double mutants which expressed only one nitrate reductase, anaerobically with glycerol as the carbon source and nitrate as the terminal electron acceptor, Potter *et al* found that only one strain, that retaining *narGHJI* grew well in these conditions. They also showed that loss of the *nar* gene resulted in enhanced growth of the Nap strain. However, in a limited nitrate environment, they found that the strain with intact Nap expression had a selective advantage.

Other pathogens, as well as *E. coli* (Stewart 1982), may also use nitrate as a terminal electron acceptor for respiration in the absence of oxygen (Lundberg, Weitzberg et al. 2004), and there may be a link between nitrate reductase activity and *Salmonella* survival in the gut (Contreras, Toro et al. 1997), which has been considered to be an anaerobic environment. Work using respiratory mutants of *E.coli* revealed that the intestinal environment also harbours microaerobic niches (Jones, Chowdhury et al. 2007). Through the construction of mutants which lacked ATP synthase, necessary for respiratory energy-conserving metabolism, Jones *et al* showed that these mutants were unable to compete with wild-type strains. Further, they found that cytochrome bo(3) oxidase, used when oxygen

tension is high, was not required for successful colonisation, whilst cytochrome bd oxidase, used when oxygen tension is low, was necessary. Colonization by nitrate and fumarate reductase deletion mutants was also adversely affected, and Jones *et al* concluded that the ability to respire both anaerobically and microaerobically was a prerequisite.

In *E. coli*, the membrane-bound nitrate reductase NarGHJI has been shown to be responsible for nitrite-induced NO production (Gilberthorpe and Poole 2008). Recently, it has been shown that a combination of sufficiency of nitrate, accumulation of nitrite and activity of the NarGHJI membrane-bound nitrate reductase in *Salmonella* results in production of both nitrous oxide (N_2O) and NO (Rowley, Hensen et al. 2012). The NarGHJI system is also expressed under high nitrate concentrations in anaerobiosis in *E. coli*(Stewart and Berg 1988; Jormakka, Richardson et al. 2004; Bertero, Rothery et al. 2005). When nitrate was limited, Rowley *et al* also showed that the periplasmic *nap* genes were preferentially expressed, resulting in little production of N_2O (Rowley, Hensen et al. 2012).

Under aerobic conditions, the *narZYWV* operon (NR-Z) was thought to be constitutively expressed in *E. coli* (Bonnefoy and Demoss 1994). Later expression studies suggest that the NR-Z operon is controlled at transcription level, and in replete media is induced 10-fold in stationary phase (Chang, Wei et al. 1999). The NR-Z system, which is an isozyme of the membrane-bound nitrate reductase enzyme encoded by the *narGHJI* operon (Blasco, Iobbi et al. 1990), is also controlled by the alternative sigma factor RpoS, upon which NR-Z is highly dependent in minimal media (Chang, Wei et al. 1999; Rychlik and Barrow 2005; Clegg, Jia et al. 2006; Jones, Chowdhury et al. 2007). Other work has concluded that narZYWV has an important role in the survival of *E. coli* under conditions of stress and starvation, in which its expression is highly regulated (Spector, Garcia del Portillo et al. 1999).

This project investigated the possibility that a link exists between nitrate, NO dissimilation and detoxification and gut occupation by *Salmonella*. The importance of nitrate reduction on gut colonization by an enteric pathogen was targeted.

The carbon substrates used preferentially by *Salmonella* also vary with changes between aerobic and anaerobic growth. Each substrate has a specific metabolic route, requiring specific enzymes, through which it is catabolised and converted to energy. The *in vitro* work in this study challenged *Salmonella* in both aerobic and anaerobic conditions, with carbon sources glucose and glycerol.

In this way, the role of the three nitrate reductase systems in *Salmonella* infection would be assessed. Previous work has already indicated that a *nap* operon deletion mutant, tested for its ability to invade epithelial cells, showed a 20-30% attenuation of invasive capability compared with SL1344 wild type (Alston *et al*., unpublished data). This suggested that the *nap* operon may have a role crucial to the early stages of *Salmonella* infection. In the case of *S*. Typhi, it has been shown that anaerobic respiration is a necessary requirement for successful invasion, and this was demonstrated in mutants unable to use either nitrate or fumarate as their terminal electron acceptor (Contreras, Toro et al. 1997).

In this chapter we investigated further not only the detoxification of a range of reactive nitrogen intermediates (RNIs), as well as the ability of *Salmonella* to dissimilate nitrate, strains of *Salmonella* lacking each and any of the three known nitrate reductase systems were constructed.

This enabled investigation of any role played by nitrate reductase enzymes in the gastrointestinal tract. The mutants were constructed using the well-established λ-Red one step gene inactivation technique (Datsenko and Wanner 2000). Some earlier work to investigate the relationships between expression levels of various genes in *S*. Typhimurium strains had already been carried out in this laboratory (Rowley, Alston, unpublished data). One interesting observation suggested that

genes of the Nap operon were co-expressed with those involved in SPI-1 synthesis, which is known to be required for *Salmonella* to successfully invade epithelial cells. It was therefore reasoned that the mutants to be tested for this project should include those components of the Nap operon involved in the construction of the periplasmic nitrate reductase. In addition, both of the other known nitrate reductase systems were deleted, comprising narGHJI and narZYWV.

4.1.2 Plasmid Purification from *E. coli*

The λ-Red system for gene knockout is an efficient way to disrupt chromosomal genes, and is highly effective in *Salmonella*. PCR primers provide the homology to the target genes, and recombination requires the phage λ recombinase, which is synthesized under the control of an inducible promoter on an easily curable, low copy number plasmid. The use of plasmids which also confer antibiotic resistance ensures that the mutants obtained can be easily isolated as antibiotic resistant colonies, by using selective plating techniques.

The template plasmids used were pKD3 and pKD4, containing chloramphenicol and kanamycin cassettes respectively. Another plasmid, pKD46, was used to provide the recombinase functions. Figure 14 illustrates the successful isolation of each of the plasmids from *E.coli*.

The plasmid pKD46 was transformed into the *Salmonella*SL1344 wild type strain by electroporation, to produce the SL1344 recipient strain which was used to construct the mutants.

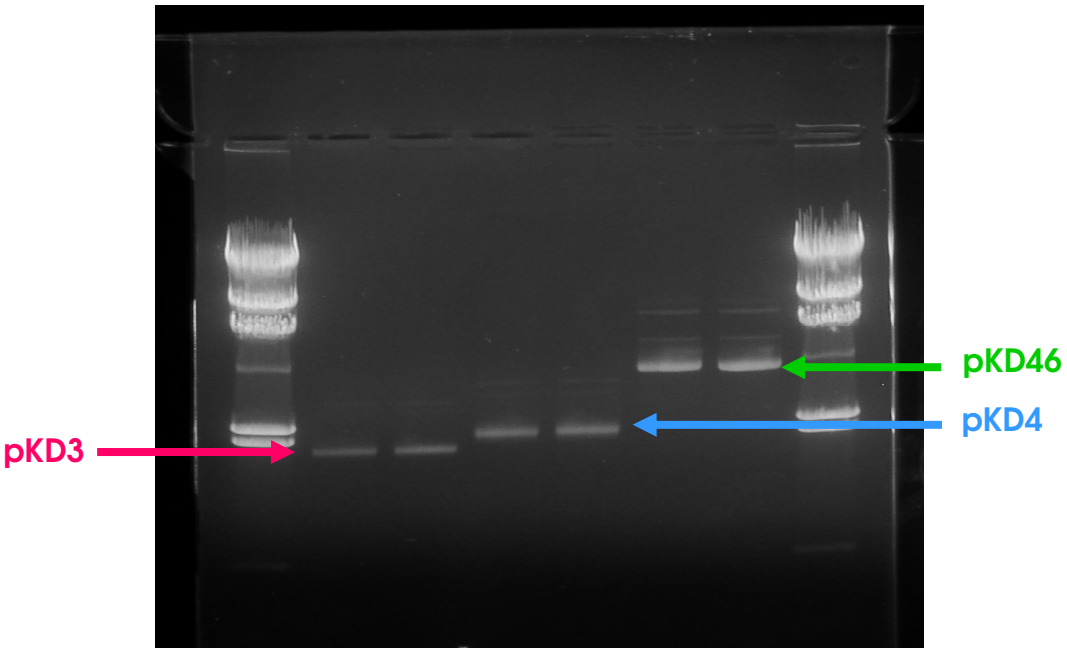
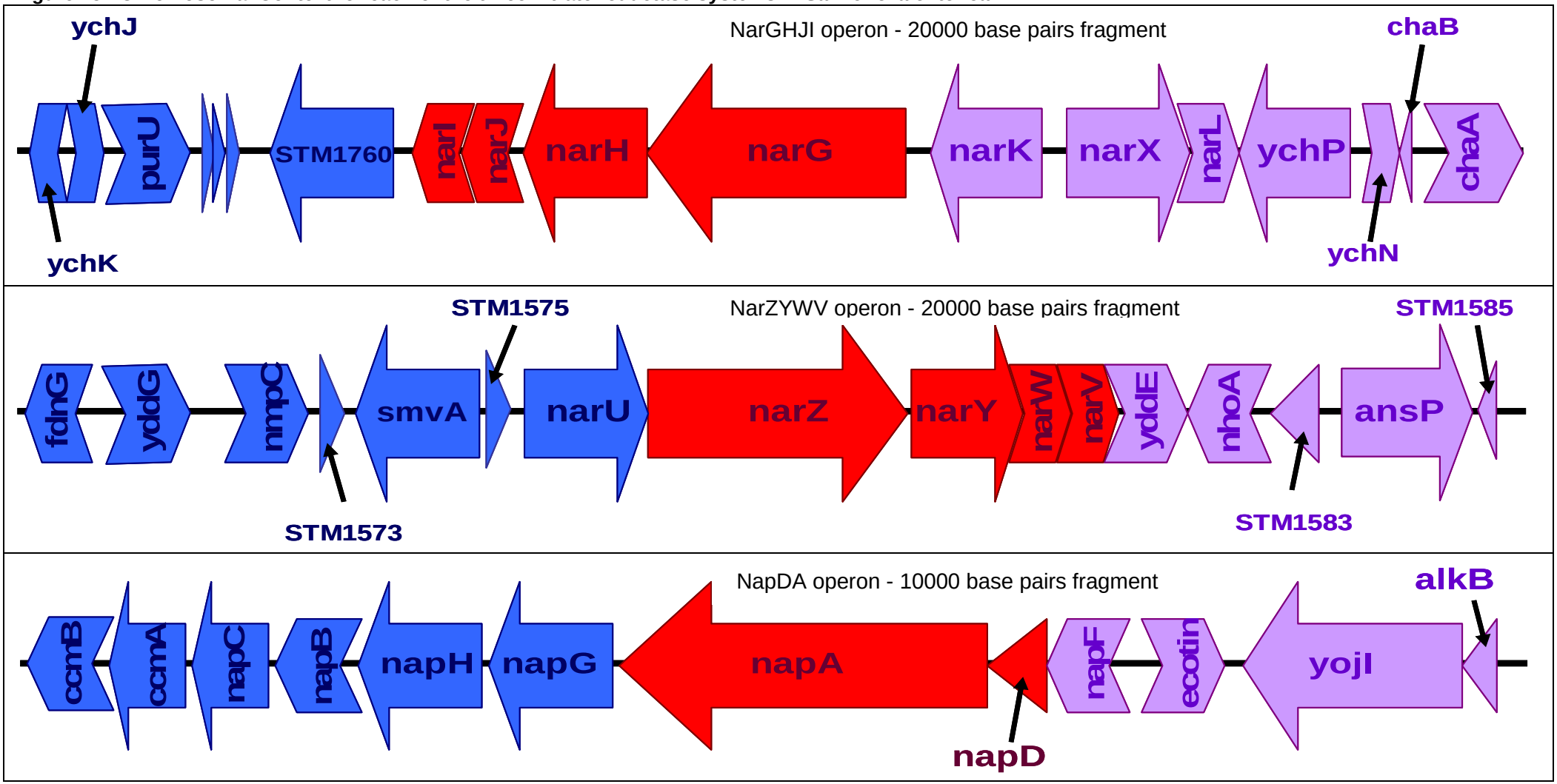


Figure 14 - Plasmids pKD3, pKD4 and pKD46 were successfully isolated from *E. coli* to be used to confer chloramphenicol and kanamycin resistance and the recombinase functions respectively during construction of the *Salmonella* mutant strains

4.1.3 Construction of gene deletions

Chloramphenicol or kanamycin resistance cassettes were amplified by PCR from plasmids pKD3 and pKD4, using primers selected for the deletion of the *narGHJI* and *narZYWV* operons, *narG*, *narZ*, *narWV* and *napDA* genes, shown in Table 2. The chromosomal context for each of the *nar* and *nap* operons is illustrated in Figure 15. The resulting PCR products were transformed by electroporation into the strain SL1344 which was harbouring the pKD46 temperature sensitive plasmid. Chloramphenicol and kanamycin resistant strains for each of the gene deletions were constructed in this way.

Figure 15 - Chromosomal Context for each of the three nitrate reductase systems in *Salmonella enterica*



4.1.4 Confirmation of gene knockouts

The deletion of the target genes and operons was verified by colony PCR, using the external primers listed in Table 2.

Figure 16 illustrates the successful construction of each of the seven deletion mutants.

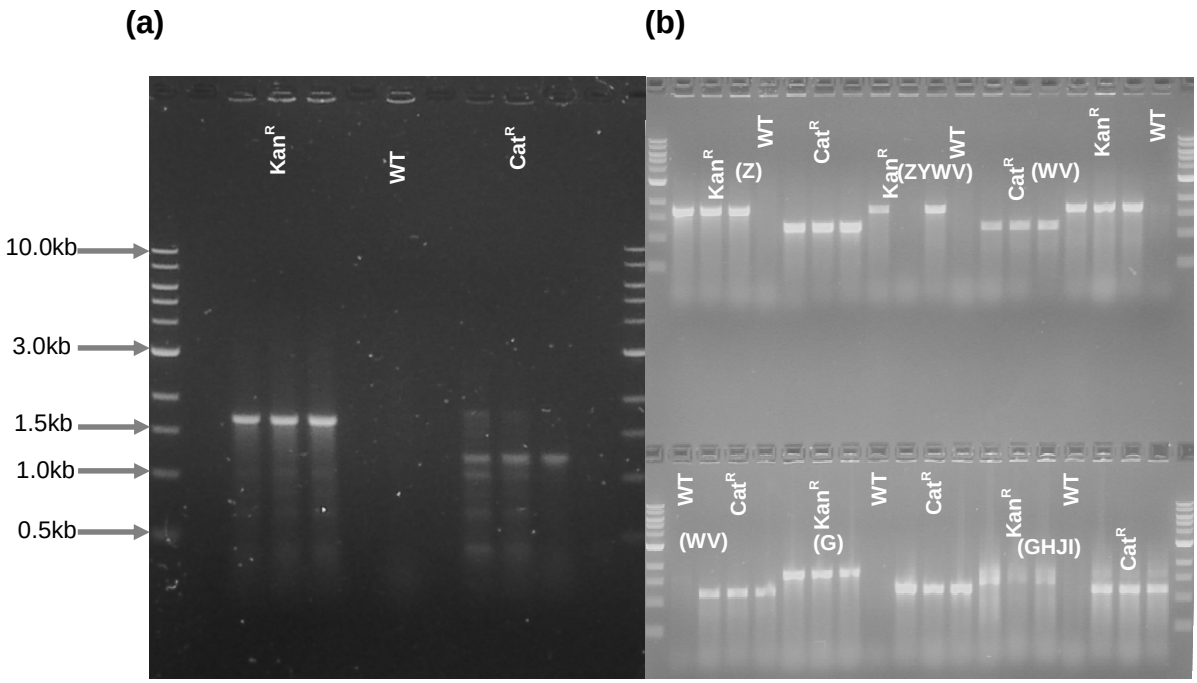


Figure 16 - PCR Confirmation of successful deletion of target genes and operon components.16(a) shows *napDA* mutants, and 16b) shows *nar* mutants. Kanamycin resistant (Kan^R) and Chloramphenicol resistant (Cat^R) mutants of each of the target genes were achieved. 'WT' indicates wild type. Three colonies per mutation were tested, along with wild type as a control.

4.1.5 Creating the double and triple deletion mutants

The double and triple deletion mutants were created by following the protocol in Section 2.5 above. Successful transductants were confirmed by double purification using two successive purifications on green indicator plates, to ensure that unstable P22 lysogens had been eradicated. PCR products were sequenced to check accuracy, although the PCR itself also confirmed the correct mutants.

4.2 Growth Curves

4.2.1 In LB and Minimal Glucose Medium (MGM)

Growth curves were carried out using nutrient sufficient media and a minimal medium containing glucose as a carbon source, both aerobically and anaerobically, to ensure that the results of future assays would reflect only any other changes or alterations made to the assay. Also, it has been shown that cells grown in Minimal Glucose Medium (MGM) have low levels of endogenous nitrite reductase activity (Mills, Rowley et al. 2008).

Figure 17 shows that in aerobic conditions and a nutrient replete medium (LB), there is no difference in growth between SL1344 wild type and the SL1344 nitrate reductase single and double mutants which had been constructed.

Similarly,in MGM in which glucose is available as the carbon source, and aerobic conditions, there was no difference in growth between SL1344 wild type and SL1344 nitrate reductase double mutants (Figure 18).

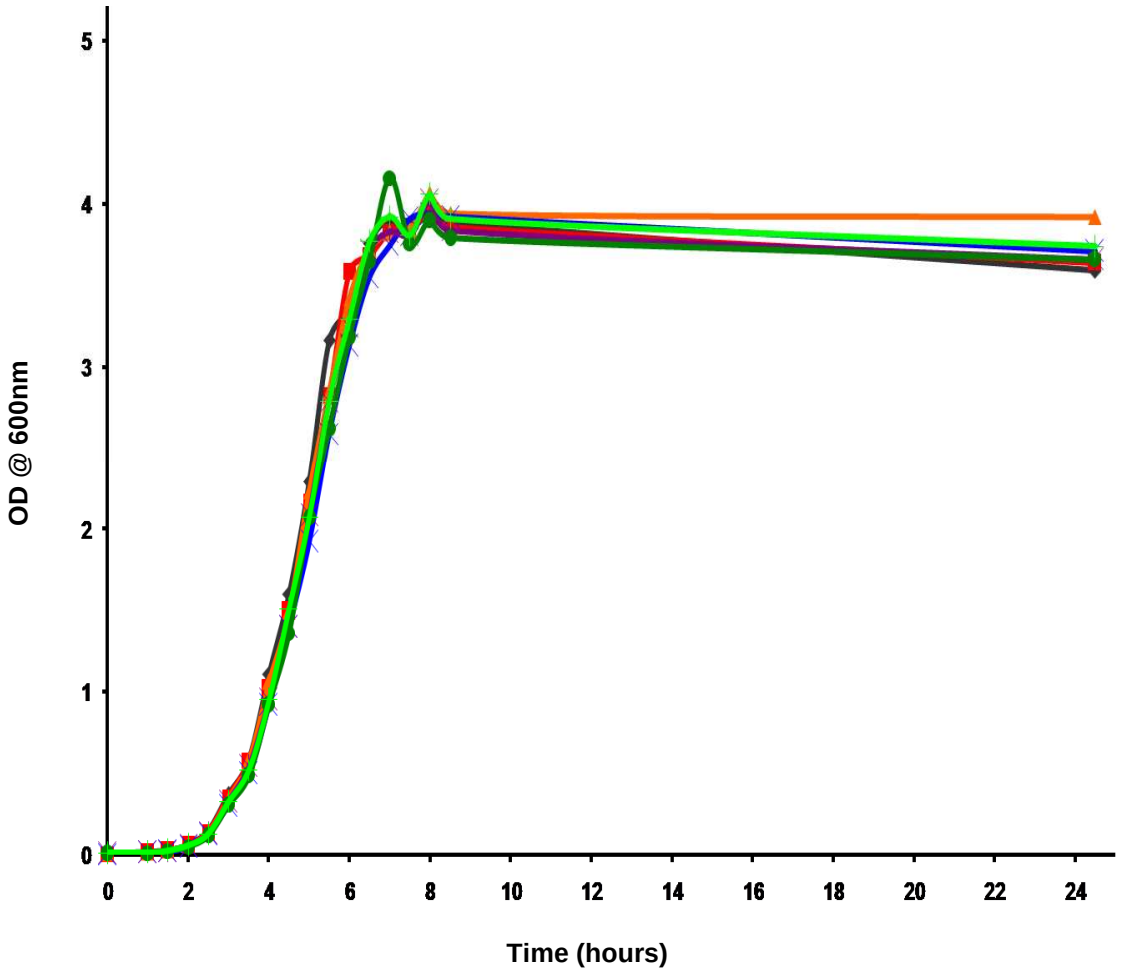


Figure 17 - Aerobic growth curve in Luria-Bertani (LB) medium, to compare SL1344 Wild Type to nitrate reductase single and double gene deletion mutants. Strains were grown in aerated conditions at 250rpm and 37℃ in LB medium, in which nutrients are replete. Absorbance was measured at $OD600_{nm}$ over a period of 24 hours.

Wild type (◆), *ΔnarG* (■), *ΔnarZ* (▲), *ΔnapDA* (×), *ΔnarGΔnapDA* (✳), *ΔnarZΔnarG* (●), *ΔnarZΔnapDA* (┼)

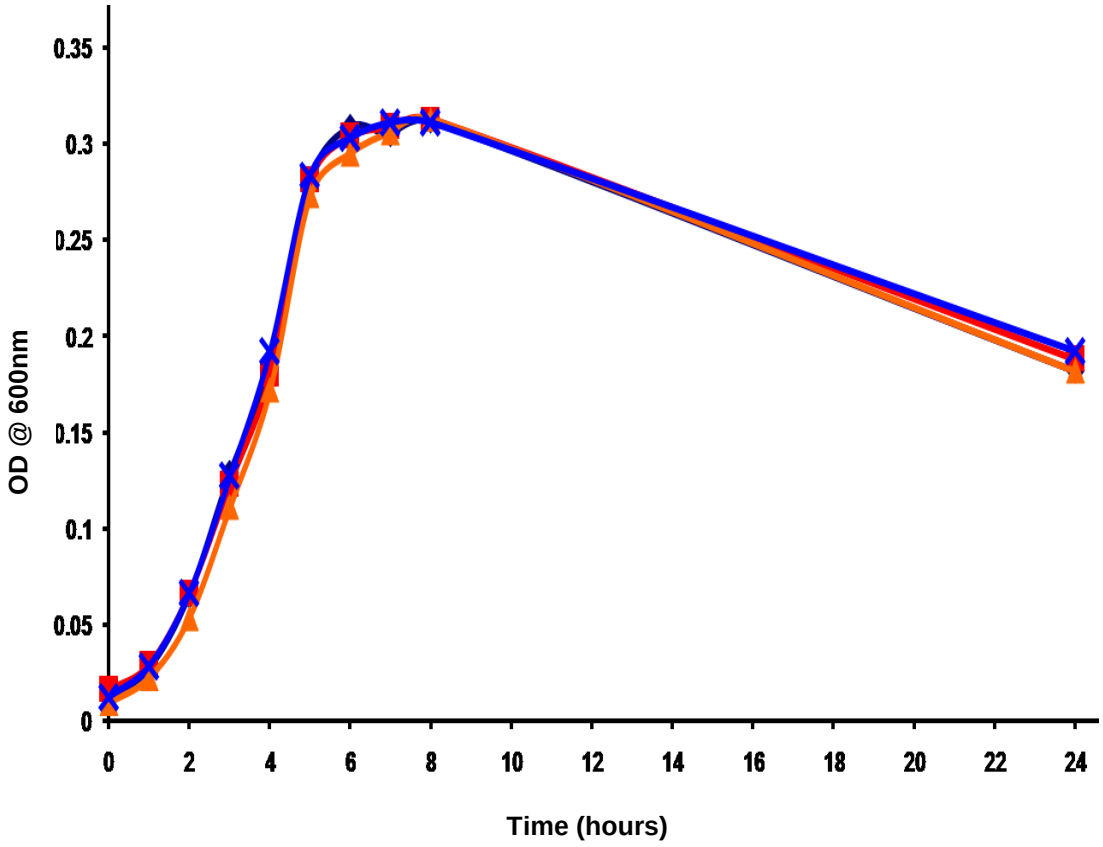


Figure 18 - Aerobic growth curve in Minimal Glucose Medium (MGM) comparing SL1344 Wild Type to nitrate reductase double gene deletion mutants. Strains were grown in aerated conditions at 250rpm and 37℃ in minimal glucose medium (MGM) in which glucose is available as the carbon source. Absorbance was measured at $OD600_{nm}$ over a period of 24 hours. There was no difference in growth between SL1344 wild type and SL1344 nitrate reductase double mutants.
Wild type (◆),*ΔnarGΔnapDA* (▲), *ΔnarZΔnarG* (■), *ΔnarZΔnapDA* (×)

4.2.2. In Glycerol Nitrate (GN) Medium, +/- Fumarate (GNF)

In aerobic growth in LB and also anaerobically in MGM, nitrate reductase deletion mutants were not growth inhibited compared to SL1344 wild type. Using glycerol as the carbon source, supplemented by nitrate and fumarate as respiratory electron acceptors, induces NrfA in *E. coli* (Darwin, Hussain et al. 1993). To further test whether the nitrate reductase mutants could grow in the presence of nitrate, the GNF medium developed in that work was adapted by removing the fumarate from it, and using anaerobic conditions.

In anaerobic growth in Glycerol Nitrate (GN) medium, there was no difference in growth between SL1344 Wild Type and SL1344 *ΔnarZYWV* and *ΔnapDA* (NR-Z and Nap respectively) nitrate reductase system single deletion mutants. In contrast however, growth of the SL1344 *ΔnarGHJI* (NR-A nitrate reductase system) single deletion mutant showed a clear reduction compared to the wild type and other two single mutants.

In a further comparison between SL1344 Wild Type and the ΔnarGHJI single and *ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutants, it was not only confirmed that the single mutant was growth inhibited, compared to the wild type, it also showed that growth of the triple deletion mutant was even more reduced (Figures 19 and 20).

In conclusion, of the single nitrate reductase deletion mutants only the growth of the NR-A *ΔnarGHJI* mutant was inhibited in this minimal medium. Since theSL1344 *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant triple nitrate reductase deletion mutant also exhibited reduced growth in this medium, and in fact grew less well than the *ΔnarGHJI* single mutant, I suggest that there are other contributory factors involved in nitrate reduction in this medium, and nitrate reductase activity is not restricted solely to the NR-A nitrate reductase system.

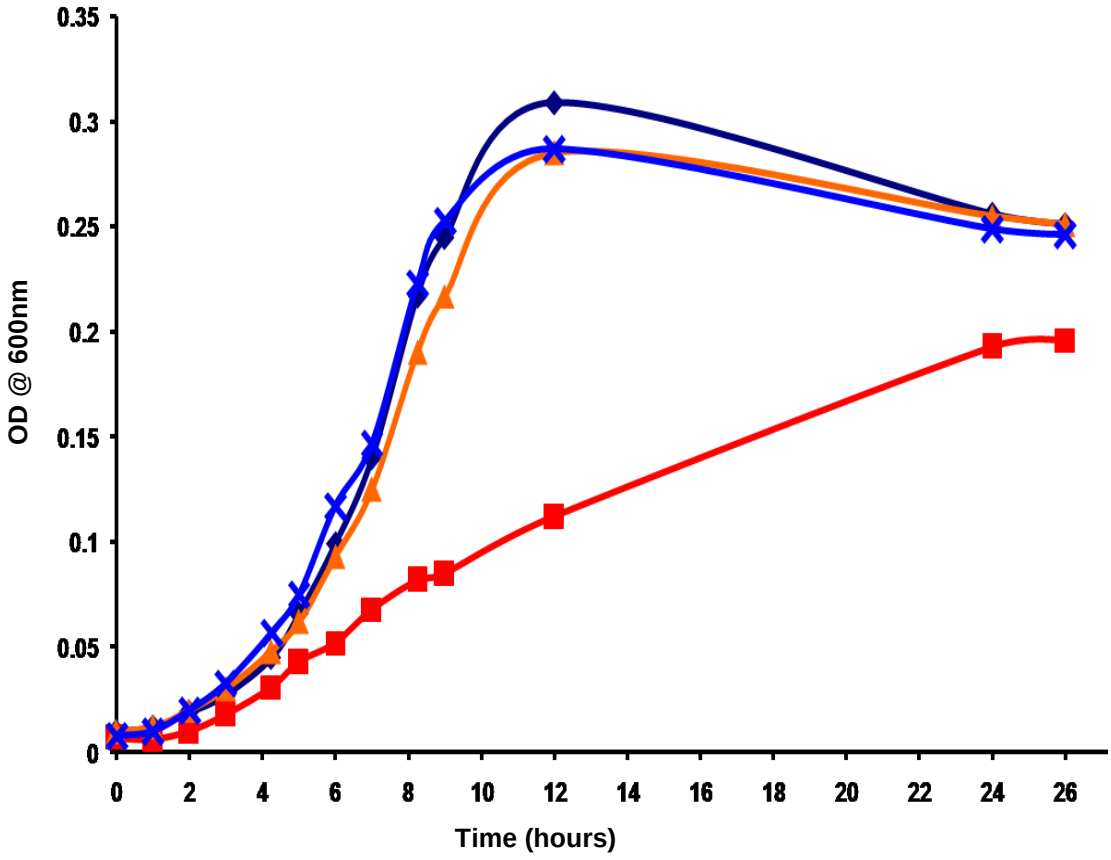


Figure 19 - Anaerobic growth curve in Glycerol Nitrate (GN) minimal medium. SL1344 Wild Type compared to SL1344 single nitrate reductase mutants Grown in GN at 37℃ and anaerobic conditions. Absorbance was measured at $OD600_{nm}$ over a period of 26 hours. Growth rate of *ΔnarGHJI* in anaerobic GN is significantly different (student t test, $p<0.05$) than the other strains. SL1344 Wild Type (◆) and SL1344 *ΔnarZYWV* (▲)and *ΔnapDA* (×) SL1344ΔnarGHJI (■).

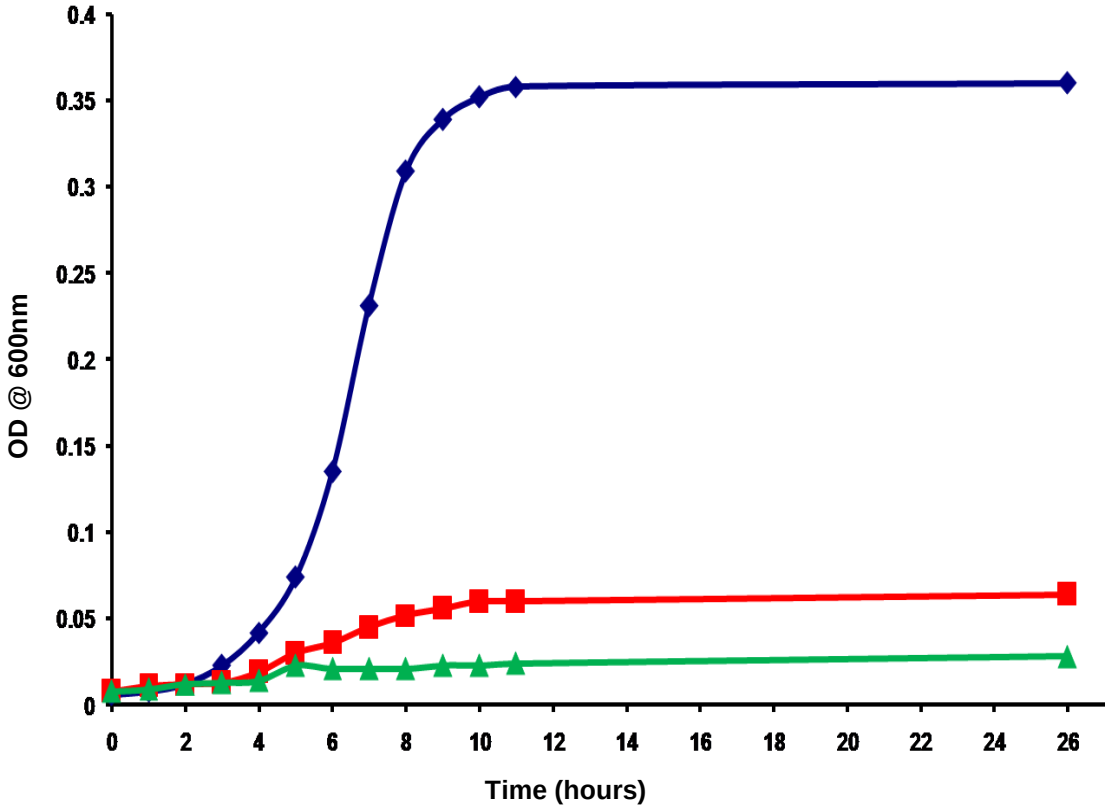


Figure 20 - Anaerobic growth curve in Glycerol Nitrate (GN) minimal medium. SL1344 Wild Type compared to ΔnarGHJI single and *ΔnarGHJIΔnarZYWVΔnapDA*. The SL1344 wild type (◆) was compared to the single SL1344 *ΔnarGHJI* (■) and the triple SL1344 *ΔnarGHJIΔnarZYWVΔnapDA* (▲) deletion mutants, in GN medium at 37℃ in anaerobic conditions. Absorbance was measured at $OD600_{nm}$ over a period of 26 hours.

In conclusion, of the single nitrate reductase deletion mutants only the NR-A *ΔnarGHJI* mutant was growth inhibited in this minimal medium. The SL1344 *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant triple nitrate reductase deletion mutant also exhibited reduced growth in this medium, and grew less than the *ΔnarGHJI* single mutant, suggesting that not only the NR-A nitrate reductase system is involved in nitrate reduction in this medium.

To explore the possibility of any contribution by the other nitrate reductase mutants to the inhibition apparent in the triple mutant, a further assay using the same conditions, with the addition of the double nitrate reductase mutants was carried out.

As is shown in Figure 21, there was no difference in growth between SL1344 Wild Type and SL1344 *ΔnarZYWVΔnapDA* (NR-Z and Nap systems) double nitrate reductase system deletion mutants. In contrast however, growth of the SL1344 *ΔnarGHJIΔnarZYWV* (NR-A and NR-Z systems) was initially reduced, but gradually recovered, though not to completely unaffected levels. Growth of the *ΔnarGHJI* single mutant was still severely curtailed compared to wild type, but the most severely affected mutants were the *ΔnarGHJIΔnapDA* (NR-A and Nap systems) double and the triple *ΔnarGHJIΔnarZYWVAΔnapDA* deletion mutants.

This result suggested that the loss of both the NR-A and Nap nitrate reductase systems together accounts for the adverse effect on growth observed in the triple nitrate reductase system deletion mutant.

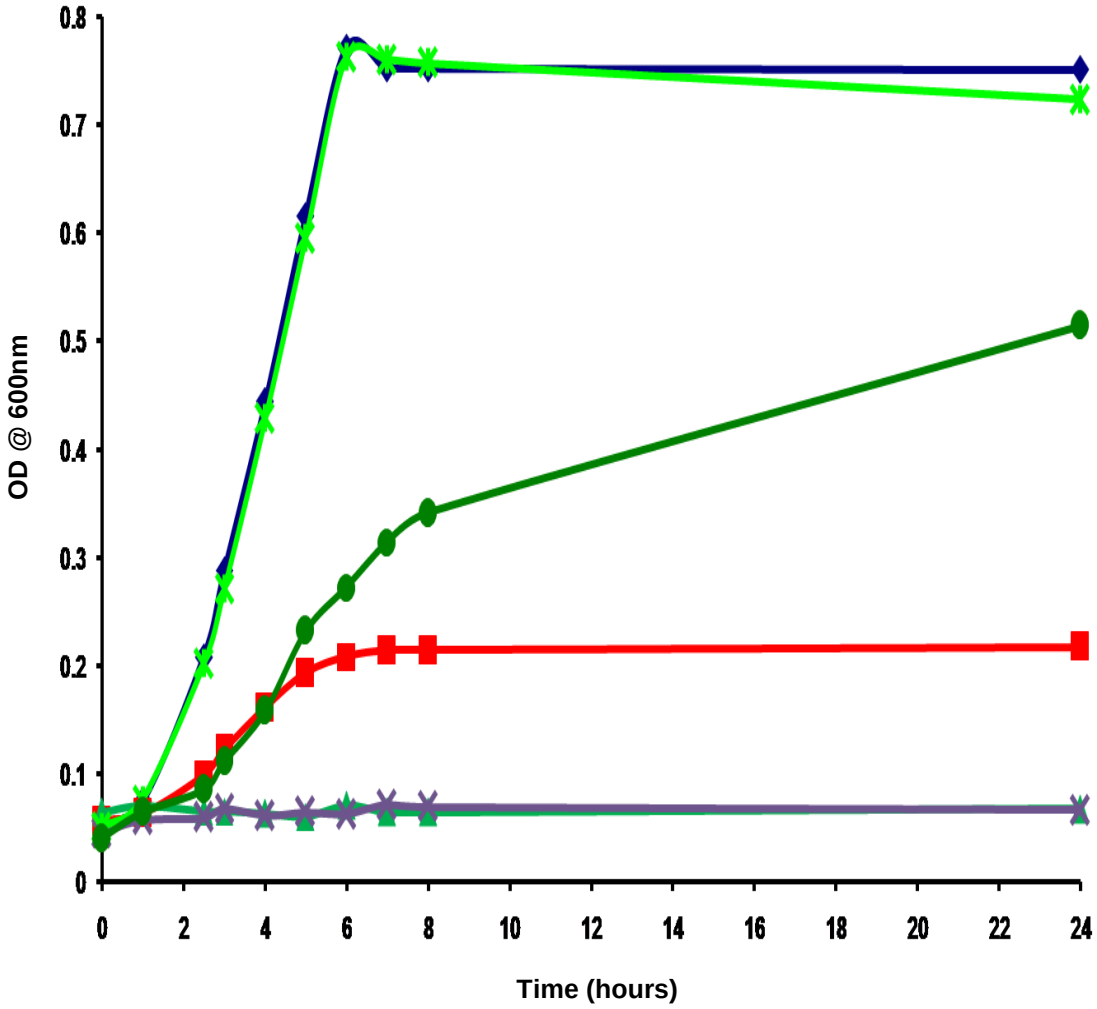


Figure 21 - Anaerobic growth in GN minimal medium, SL1344 Wild Type compared to ΔnarGHJI single, all double and the triple nitrate reductase system mutants. Grown in GN at 37℃ in anaerobic conditions, over a 24-hour period, SL1344 Wild Type (◆), SL1344*ΔnarZYWVΔnapDA* (✳**)**, SL1344*ΔnarGHJIΔnarZYWV* (●),SL1344*ΔnarGHJI* single mutant (■), SL1344*ΔnarGHJIΔnapDA* (✕) SL1344*ΔnarGHJIΔnarZYWVAΔnapDA* (▲) deletion mutants. Absorbance was measured at $OD600_{nm}$. Growth rate of *ΔnarGHJIΔnarZYWV*, *ΔnarGHJI*, *ΔnarGHJIΔnarZYWVAΔnapDA* in anaerobic GN are significantly different (student t test, $p<0.05$) to WT.

While growth of the SL1344 *ΔnarZYWVΔnapDA* (NR-Z, Nap nitrate reductase system) double mutant matches that of wild type, anaerobically in GN medium, growth of the SL1344 *ΔnarGHJIΔnarZYWV* double mutant is initially inhibited but later recovers, although without reaching similar levels as the wild type strain. Deletion of the *ΔnarGHJI* NR-A system alone did not inhibit growth to the same extent as the loss of both the NR-A and Nap systems in the *ΔnarGHJIΔnapDA* double mutant, in which growth was as severely curtailed as that of the triple *ΔnarGHJIΔnarZYWVAΔnapDA* deletion mutant. Since growth of the NR-Z, Nap nitrate reductase mutant was unaltered from that achieved by the wild type, I suggest that the complete loss of growth in the triple mutant is in fact due to the loss of the NR-A and Nap systems, and not loss of the NR-Z system.

To test whether substitution with a different electron acceptor would rescue growth in the mutants which showed growth inhibition in the GN media, Fumarate was added to the medium.

Figure 22 shows that when grown anaerobically in GN minimal medium, with the addition of Fumarate as a replacement electron acceptor, there was no difference in growth between SL1344 Wild Type and SL1344 *ΔnarZYWVΔnapDA* (NR-Z and Nap systems) double nitrate reductase system deletion mutant. In contrast however, growth of the SL1344 *ΔnarGHJIΔnarZYWV* (NR-A and NR-Z systems) double deletion mutant was initially reduced, but gradually recovered, though not to completely unaffected levels. Growth of the *ΔnarGHJI* single deletion mutant (NR-A system only) was still severely curtailed compared to wild type, but the most severely affected mutants were the *ΔnarGHJIΔnapDA* (NR-A and Nap systems) double and the triple *ΔnarGHJIΔnarZYWVAΔnapDA* deletion mutants. Clearly, the addition of fumarate did not rescue growth in those mutants.

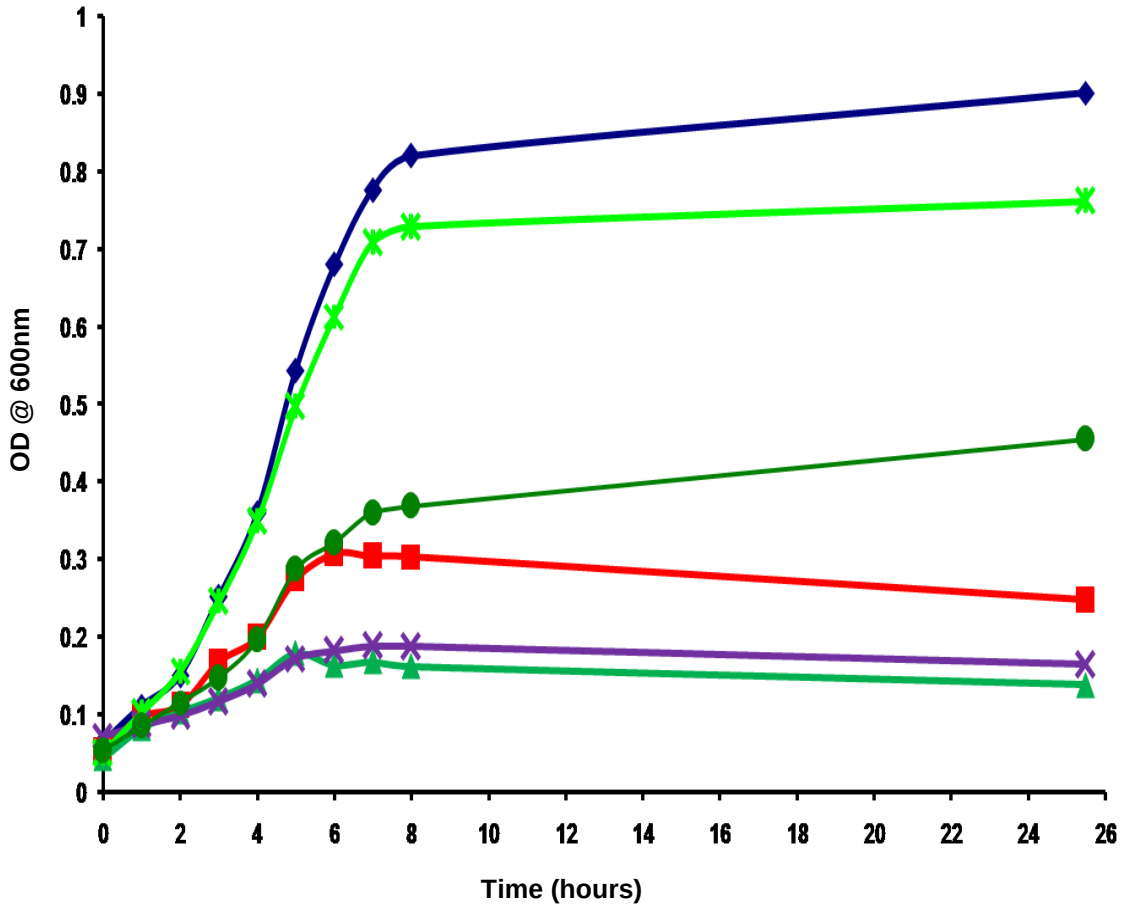


Figure 22 – Anaerobic growth in GN minimal medium, SL1344 Wild Type compared to ΔnarGHJI single, all double and the triple nitrate reductase system mutants with the addition of Fumarate as a replacement electron acceptor to the GN minimal medium. Grown in GN + Fumarate at 37℃ in anaerobic conditions. SL1344 Wild Type (◆), SL1344*ΔnarZYWVΔnapDA* (✳), SL1344*ΔnarGHJIΔnarZYWV* (●), SL1344*ΔnarGHJI* single mutant (■), SL1344*ΔnarGHJIΔnapDA* (✕), SL1344 *ΔnarGHJIΔnarZYWVAΔnapDA* (▲). Absorbance was measured at $OD600_{nm}$. Growth rate of *ΔnarGHJI* single mutant, *ΔnarGHJIΔnapDA, ΔnarGHJIΔnarZYWVAΔnapDA*, in GNF are significantly different (student t test, $p<0.05$)to WT.

In order to characterize and summarize the growth profiles of all of the nitrate reductase system mutants, single and double system deletions as well as the triple nitrate reductase deletion mutant, in the presence of nitrate, a series of anaerobic growth curves using GN minimal medium and without the addition of fumarate was performed.

For Figure 23, the growth was observed over a 24 hour period. No difference was apparent between the wild type and *ΔnarZYWV* single deletion mutant. The *ΔnapDA* single deletion mutant and the *ΔnarZYWVΔnapDA* double deletion mutant grew at a similar rate as wild type for 10 hours, but thereafter, the decline in bacterial density occurred more quickly than for the wild type. In the case of the *ΔnarGHJI* single deletion mutant and the *ΔnarGHJIΔnarZYWV* double deletion mutant, growth was considerably curtailed over the initial 10 hour period. However, growth also continued for the next 14 hours, albeit at a very slow rate. Growth was most severely inhibited in the *ΔnarGHJIΔnapDA* double deletion mutant, and the triple *ΔnarGHJIΔnarZYWVΔnapDA* deletion mutant. This suggests that the loss of the *narGHJI* operon and the *napDA* operon accounts for most if not all of the effect observed in the triple mutant. In contrast, loss of the *narZYWV* operon makes little if any contribution to inhibiting the growth of *Salmonella* in a minimal medium containing nitrate.

To ascertain whether there was any further difference in the growth pattern, it was decided to carry out a similar assay over twice this time period, and the result of those assays is shown in Figure 24. Over two days, the growth pattern for the wild type and the *ΔnarZYWVΔnapDA* double deletion mutant were very similar. Over the first twelve hours, the growth for the *ΔnarZYWV* and *ΔnapDA* single deletion mutants were very similar to each other, although markedly slower than for the wild type and the *ΔnarZYWVΔnapDA* double deletion mutant. However, between 12 and 36 hours, the differences between these four strains

were lost so that after 48 hours there is no significant difference between the bacterial densities in any of them.

Noticeably, the other four mutants (*ΔnarGHJI* single deletion mutant, the *ΔnarGHJIΔnarZYWV* and the *ΔnarGHJIΔnapDA* double deletion mutants and the *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant)all failed to thrive or grow. This confirms that of the three nitrate reductase systems, *narGHJI* has the major effect on growth in minimal medium containing nitrate.

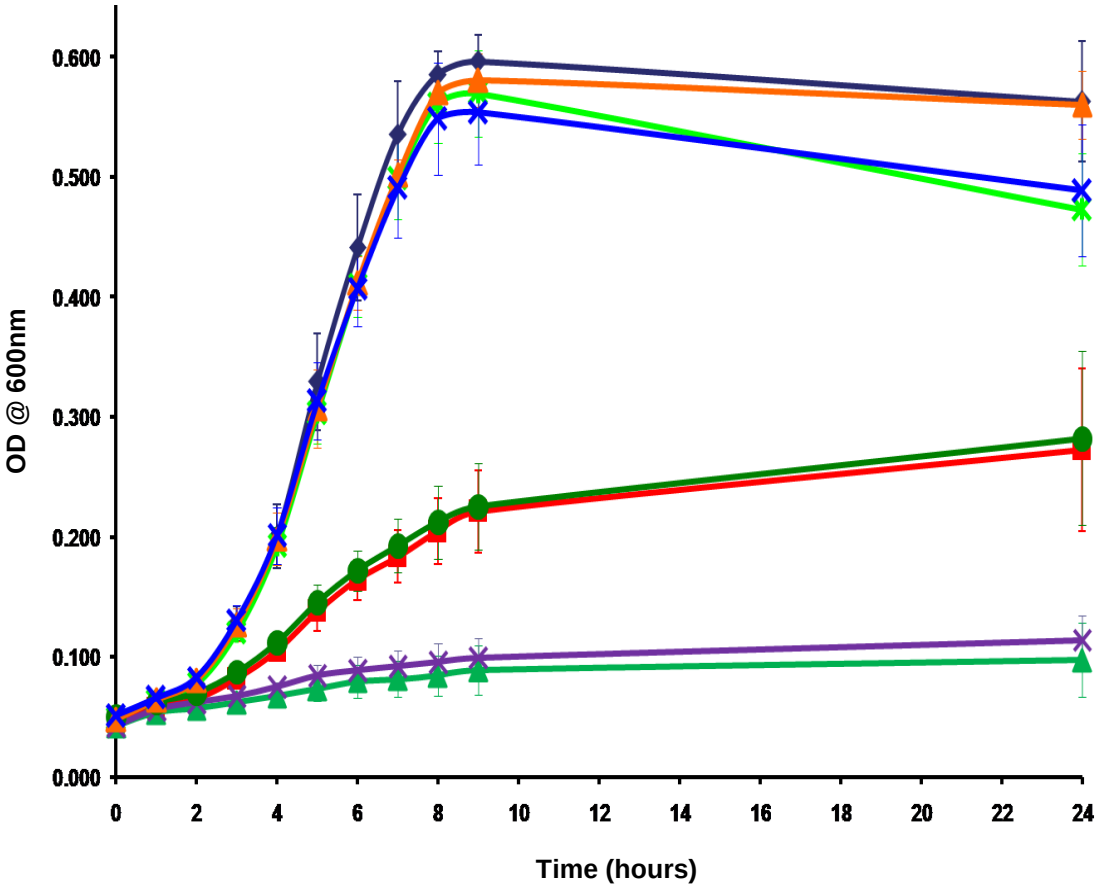


Figure 23 - Anaerobic growth over 24 hours in GN medium of *Salmonella* SL1344 wild type compared to single, double and triple nitrate reductase mutants.Wild type(◆), SL1344*ΔnarZYWV*(▲),SL1344*ΔnapDA* (×), SL1344*ΔnarGHJI*(■), SL1344*ΔnarZYWVΔnapDA* (✳), SL1344*ΔnarGHJIΔnarZYWV* (●), SL1344*ΔnarGHJIΔnapDA* (×), SL1344*ΔnarGHJIΔnarZYWVnapDA* (▲). Absorbance was measured at $OD600_{nm}$. Final OD of *ΔnarZYWVΔnapDA*, *ΔnarGHJIΔnapDA*, *ΔnarGHJI*, *ΔnarGHJIΔnarZYWVΔnapDA* mutants significantly different (student t test, $p<0.05$) to WT in GN medium after 24 hrs. Error bars show the Standard Error of the Mean for three separate experiments.

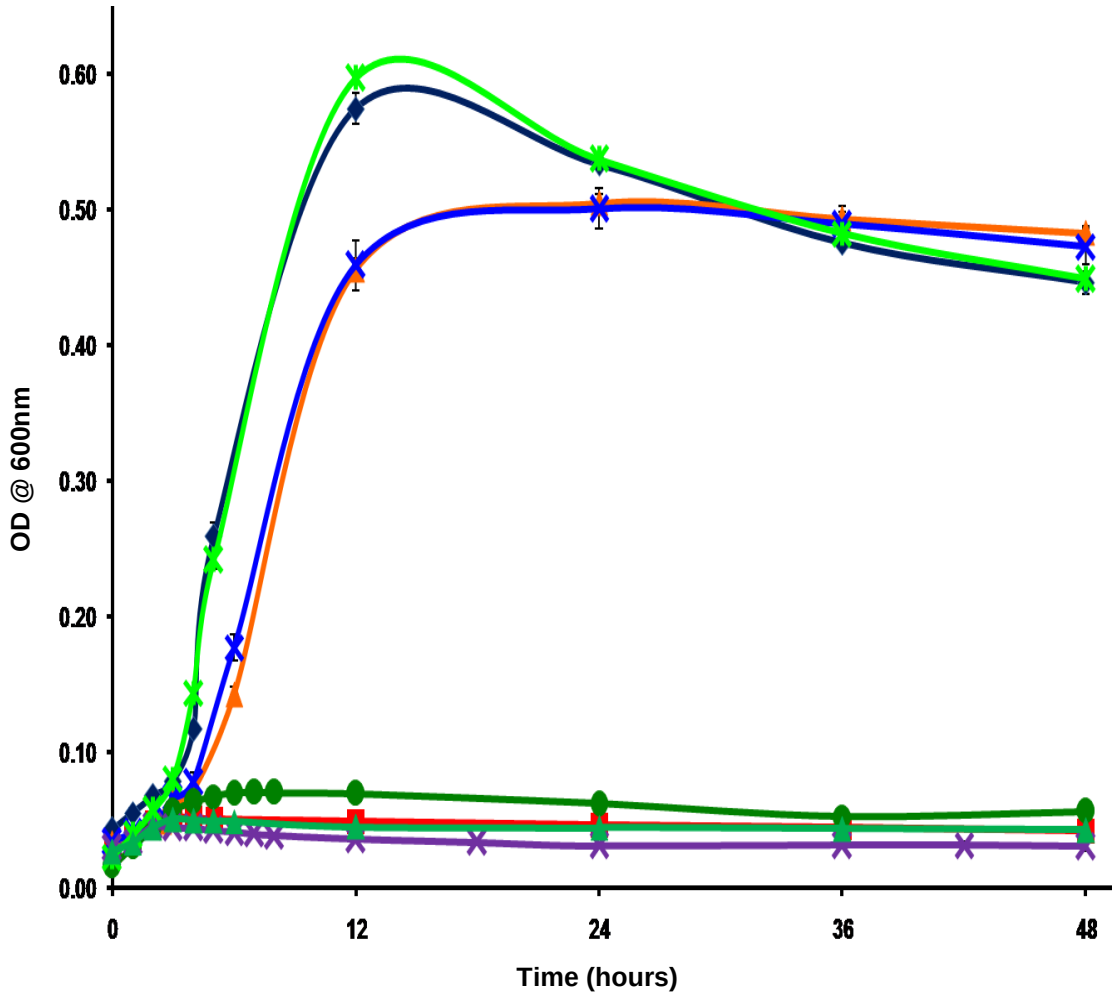


Figure 24 - Anaerobic growth in GN medium over 48 hours of *Salmonella* wild type compared to single, double and triple nitrate reductase deletion mutants. SL1344 wild type (◆), SL1344*ΔnarZYWV* (▲), SL1344*ΔnapDA* (✕), SL1344*ΔnarGHJI* (■), SL1344*ΔnarZYWVΔnapDA*(✳), SL1344 *ΔnarGHJIΔnarZYWV* (●), SL1344 *ΔnarGHJIΔnapDA* (✕), SL1344 *ΔnarGHJIΔnarZYWVΔnapDA*(▲). Absorbance was measured at $OD600_{nm}$. Final OD of *ΔnarGHJI* single deletion mutant, the *ΔnarGHJIΔnarZYWV* and the *ΔnarGHJIΔnapDA* double deletion mutants and the *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant mutants significantly different (student t test, $p<0.05$) to WT in GN medium after 48 hrs. Error bars show the Standard Error of the Mean for three separate experiments.

.

4.2.3 In MM5.8 Minimal Medium

Since *Salmonella* is able to survive and replicate within macrophages, we wanted to test the ability of nitrate reductase mutants to grow in this environment. It was necessary to assess the fitness of the nitrate reductase mutants which had been constructed to grow and replicate in a medium which closely mimics the intracellular environment of the *Salmonella* Containing Vacuole (SCV) within macrophages.It has been shown that the internal environment of SCVs is acidic, and concluded that this acidity is in fact a requirement for *Salmonella* to successfully replicate within macrophages (Rathman, Sjaastad et al. 1996). This finding has been further refined to show that the assembly of the SPI-2 TTSS apparatus is dependent on an acidic environment (Beuzon, Banks et al. 1999; Rappl, Deiwick et al. 2003; Xu and Hensel 2010). The medium used was MM5.8, a minimal medium adjusted to pH5.8. The macrophage environment is likely to be also at least microaerobic, since it is bathed in the serum of oxygenated blood.Therefore, the assay was carried out in physiologically relevant conditions at 37℃ and 5% CO_2, and the results of the growth assay carried out in this aerated medium are shown in Figure 25.

In fact, there was no difference between the growth patterns observed for the SL1344 single, double or triple nitrate reductase mutant when compared to the SL1344 wild type strain in MM5.8 medium.

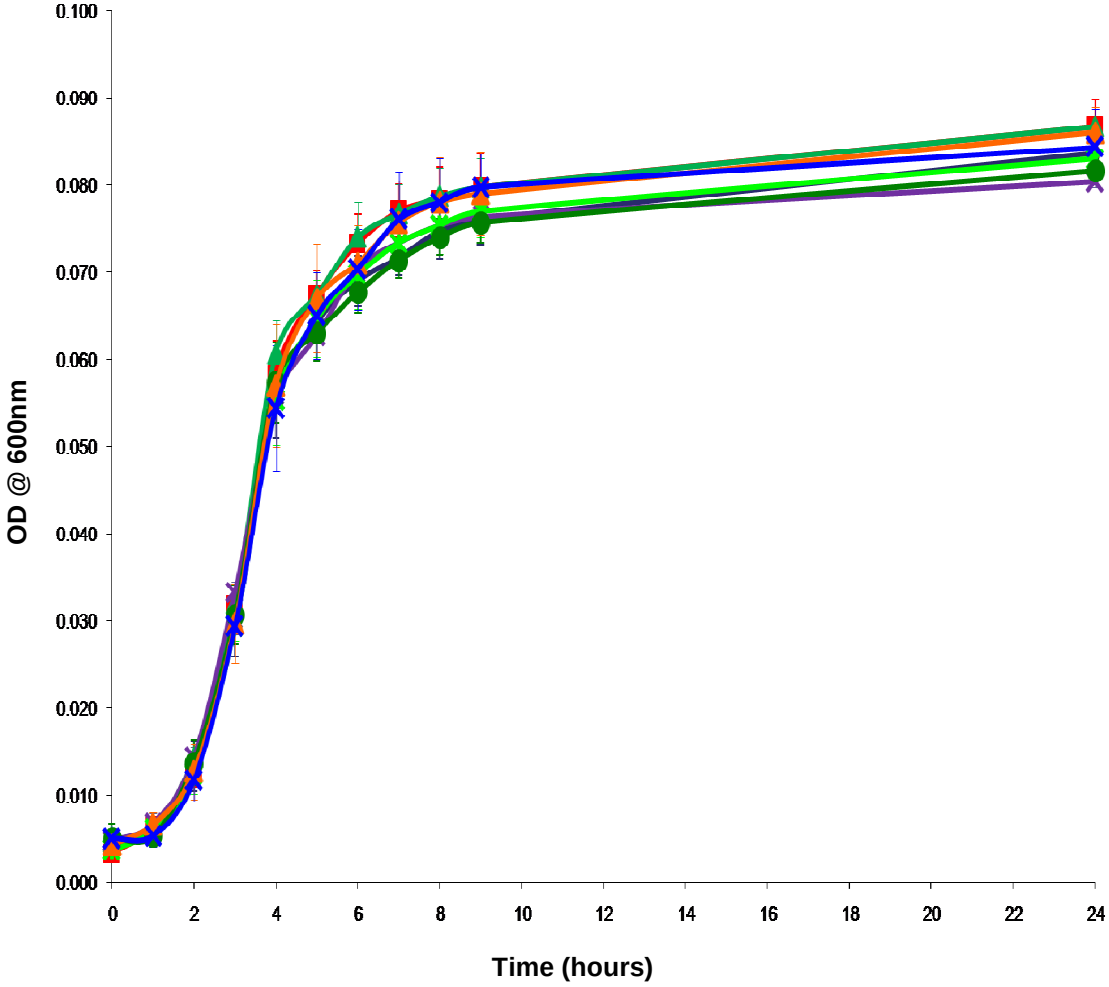


Figure 25 - Aerobic growth in MM5.8 medium, of *Salmonella* SL1344 wild type compared to SL1344 single, double and triple nitrate reductase deletion mutants. SL1344 wild type (◆), SL1344*ΔnarGHJI* (■), SL1344*ΔnarZYWV* (▲), SL1344*ΔnapDA* (×),SL1344*ΔnarGHJIΔnarZYWV* (•), SL1344*ΔnarGHJIΔnapDA* (×), SL1344*ΔnarZYWVΔnapDA* (✳), SL1344*ΔnarGHJIΔnarZYWVnapDA* (▲). Absorbance was measured at $OD600_{nm}$. Error bars show the Standard Error of the Mean for three separate experiments.

4.3 Motility Assays

As mentioned previously, (Section 1.6.1), *Pseudomonas aeruginosa* contains a *narK1K2GHJI* operon which encodes two nitrate/nitrite transporters and a nitrate reductase (Schreiber, Krieger et al. 2007). Other work has revealed that *nar* mutants in *P. aeruginosa* also suffer motility and swarming defects (Van Alst, Picardo et al. 2007). In *E.coli*, a*Δhmp* deletion mutant fails to show swarming motility (Stevanin *et* al, 2007). Other work has also shown that virulence of a nitrate reductase mutant of *P. aeruginosa* is attenuated in *Caenorhabditis elegans* (Van Alst, Picardo et al. 2007), which further strengthens the link between nitrate metabolism and motility and pathogenesis in this opportunistic human pathogen. In order to explore this aspect of the characteristics of the nitrate reductase mutants which have been constructed in *Salmonella*, motility assays using minimal medium 0.4% agar plates were carried out to test whether *nar* mutants in *Salmonella* would be similarly affected by the loss of one, all, or any combination of the nitrate reductase systems.

The results of these assays are illustrated in Figure 26. In contrast to the findings reported for *P. aeruginosa* nitrate reduction mutants (Van Alst, Picardo et al. 2007) and *E. coli Δhmp* deletion mutant (Stevanin, Read et al. 2007), mutation of the nitrate reductase systems does not affect motility of *S*. Typhimurium.

In *P. aeruginosa*, the production of the biosurfactant rhamnolipid or its precursor hydroxyalkanoyloxy alkanoic acid, is essential to the ability of the bacterium to adopt swarming motility, and this is in turn dependant on the presence of a good nitrogen source in the form of nitrate (Van Alst, Picardo et al. 2007). It is this mechanism which has been proposed as the relevant one in the observation of this effect on motility in *P. aeruginosa* which arises when the nitrate reductase enzymes have been compromised by mutation.

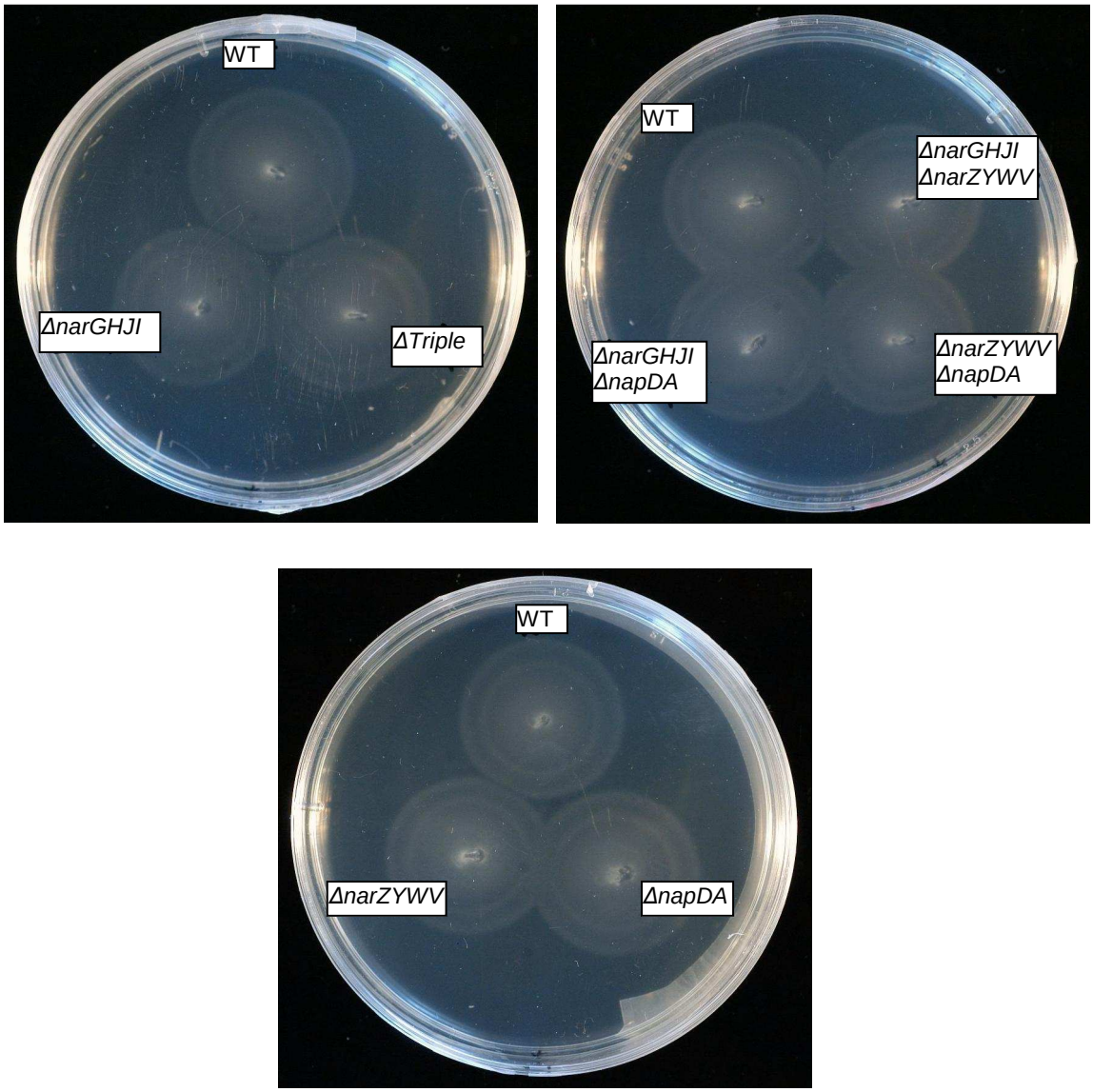


Figure 26 - **Motility Assay to compare the ability of SL1344 Wild Type and each of the nitrate reductase single, double and triple mutants to remain motile in 0.4% agar.** Differences in the number of bacteria inoculated to the plates was allowed for, and no difference in the motility of the bacteria was demonstrable. 'WT' denotes SL1344 wild type samples.

4.4 Discussion

To facilitate characterisation of nitrate reductases in *Salmonella*, single, double and triple nitrate reduction deletion mutants were constructed. A combination of colony PCR and PCR analysis confirmed that the intended mutations had been induced in the *Salmonella* strains to be tested further, by both *in vitro* and *in vivo* methods. Some of the mutants produced have also been used successfully in another project (Rowley, Hensen et al. 2012).

In either nutrient replete medium (LB) or minimal medium containing glucose (MGM), the growth of SL1344 nitrate reductase mutants is similar to that of SL1344 wild type, regardless of whether growth proceeds aerobically or anaerobically. In aerobic conditions, it was demonstrated that in LB, there was no growth difference between SL1344 wild type and the SL1344 nitrate reductase single and double mutants which had been constructed. Furthermore, there was no growth difference between SL1344 wild type and the SL1344 nitrate reductase single and double mutants given aerobic conditions and MGM. Even in anaerobic conditions which we might have expected to present a greater challenge to growth in the mutants, we observed no significant growth differences between SL1344 Wild Type and the *ΔnarGHJI* nitrate reductase single deletion mutant. The addition of 40μM NO also exerted no effect on the NR-A operon deletion mutant. This is what would have been expected, since a nitrate reductase would be involved in the production of nitrite and subsequently nitric oxide, rather than their detoxification. It has recently been confirmed that both the membrane bound nitrate reductase (Nar) and the periplasmic nitrate reductase (Nap) are instrumental in the production of both NO and nitrous oxide (N_2O) (Rowley, Hensen et al. 2012). Other work has shown that use of glycerol rather than glucose as the carbon source and supplemented with nitrate and fumarate induced expression of *nrfA* in *E.coli* (Darwin, Hussain et al. 1993). In *E.coli*, NrfA

requires fumarate, since it is the terminal reductase of the formate-dependent pathway of nitrite reduction to ammonia (Darwin, Hussain et al. 1993). However, supplementation of this respiratory electron acceptor is not required for nitrate reductases, pathways for which are not formate dependent. Consequently, to assess the ability of the nitrate reductase mutants to grow in the presence of replete nitrate, the Glycerol Nitrate Fumarate (GNF) medium developed by Darwin *et al* was adapted, to remove the fumarate, and then tested against the nitrate reductase mutants in anaerobic conditions. Although no difference in growth betweenSL1344 Wild Type and SL1344 *ΔnarZYWV* and *ΔnapDA* (NR-Z and Nap respectively) nitrate reductase system single deletion mutants was established, it was interesting that the SL1344 *ΔnarGHJI* (NR-A nitrate reductase system) single deletion mutant consistently and repeatedly showed a significant growth reduction compared to the wild type and other two single mutants. Work elsewhere has found that a *narG* mutant of *M. tuberculosis* also fails to grow in nitrate replete broth media (Malm, Tiffert et al. 2009). Before speculating on what the reasons for this difference might be, comparison was also made between SL1344 Wild Type and the *ΔnarGHJI* single and *ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutants. This confirmed the growth inhibition of the single mutant, and also demonstrated that growth of the triple deletion mutant was even more reduced. The fact that the triple deletion mutant was even more growth inhibited than the single *ΔnarGHJI* mutant suggested that not only the NR-A nitrate reductase system is involved in nitrate reduction in this medium. This idea was tested by carrying out further assays using double nitrate reductase mutants. There was no difference in growth between SL1344 Wild Type and SL1344 *ΔnarZYWVΔnapDA* (NR-Z and Nap systems) double nitrate reductase system deletion mutant. In contrast however, growth of the SL1344 *ΔnarGHJIΔnarZYWV* (NR-A and NR-Z systems) was initially reduced, and gradually recovered, though not to completely unaffected levels. Growth of the

ΔnarGHJI single mutant was still severely curtailed compared to wild type, but the most severely affected mutants were the *ΔnarGHJIΔnapDA* (NR-A and Nap systems) double and the triple *ΔnarGHJIΔnarZYWVAΔnapDA* deletion mutants. From these results, it was possible to conclude that loss of both the NR-A and Nap nitrate reductase systems together inhibits growth almost completely, and accounts for the entire adverse effect on growth observed in the triple nitrate reductase system deletion mutant. These findings agree with those of earlier work on *M. tuberculosis* and *E.coli* in which it was shown that NarGHJI mutants were disadvantaged in replete nitrate under anaerobic conditions (Sohaskey 2008; Malm, Tiffert et al. 2009). Growth of the SL1344 *ΔnarZYWVΔnapDA* (NR-Z, Nap nitrate reductase system) double mutant matches that of wild type, anaerobically in GN medium. However, growth of the SL1344 *ΔnarGHJIΔnarZYWV* double mutant is initially inhibited but later recovers, although without reaching similar levels as the wild type strain. This suggests that when Nap is present in the absence of NR-Z, expression of Nap is upregulated. Still, while the NR-A system could compensate for the loss of both the NR-Z and Nap systems, this was not reciprocal as the Nap system did not compensate completely for the loss of the NR-A system. This suggests that the complete loss of growth in the triple mutant is in fact due to the loss of the NR-A and Nap systems, and not loss of the NR-Z system. To elucidate whether availability of another electron acceptor would rescue growth in the mutants which showed growth inhibition in the GN media, fumarate was added to the medium. It was concluded that the addition of fumarate made no difference to the previous results and did not rescue growth in NR-A and Nap mutants.

Growth profiles of all of the nitrate reductase system mutants which had been constructed were assessed, in nitrate replete anaerobic conditions, using GN minimal medium. Over a 24 hour period, it was established that there was no discernible difference between the growth of the wild type and *ΔnarZYWV* single

deletion mutant. The *ΔnapDA* single deletion mutant and the *ΔnarZYWVΔnapDA* double deletion mutant grew at a similar rate as wild type for 10 hours, but thereafter, the decline in bacterial density occurred more quickly than for the wild type. In the case of the *ΔnarGHJI* single deletion mutant and the *ΔnarGHJIΔnarZYWV* double deletion mutant, growth was considerably curtailed over the initial 10 hour period. However, growth also continued for the next 14 hours, albeit at a very slow rate. Growth was most severely inhibited in the *ΔnarGHJIΔnapDA* double deletion mutant, and the triple *ΔnarGHJIΔnarZYWVΔnapDA* deletion mutant. This suggests that the loss of the *narGHJI* operon and the *napDA* operon accounts for most if not all of the effect observed in the triple mutant, and concurred with the earlier finding. In contrast, loss of the *narZYWV* operon makes little if any contribution to inhibiting the growth of *Salmonella* in a minimal medium containing nitrate.

To ascertain any effects of an extra time period with replete nitrate, a similar assay was done over twice the initial time period. Over two days, the growth pattern for the wild type and the *ΔnarZYWVΔnapDA* double deletion mutant were very similar. Over the first twelve hours, the growth for the *ΔnarZYWV* and *ΔnapDA* single deletion mutants were very similar to each other, although markedly slower than for the wild type and the *ΔnarZYWVΔnapDA* double deletion mutant. However, between 12 and 36 hours, the differences between these four strains were lost so that after 48 hours there is no significant difference between the bacterial densities in any of them.

Noticeably, the other four mutants (*ΔnarGHJI* single deletion mutant, the *ΔnarGHJIΔnarZYWV* and the *ΔnarGHJIΔnapDA* double deletion mutants and the *ΔnarGHJIΔnarZYWVΔnapDA* triple deletion mutant)all failed to thrive or grow. This also confirmed that of the three nitrate reductase systems, *narGHJI* has the major effect on growth in minimal medium replete with nitrate.

As *Salmonella* survives the harsh environment within mammalian macrophages, the fitness of the nitrate reductase mutants to grow and replicate in a medium which closely mimics the intracellular environment of the *Salmonella* Containing Vacuole (SCV) within macrophages was tested. Many intracellular pathogens are specially adapted to withstand the rigours of the harsh environment found within macrophages (Lavigne, O'Callaghan et al. 2005), and particularly with in the phagosome itself. Indeed, several, such as *Toxoplasma, Leishmania, Mycobacterium*, *Legionella* and including *Salmonella spp*., are exquisitely adapted to actually thrive in such surroundings (Amer and Swanson 2002). The phagosome content is mildly acidic and also low in magnesium ions (Mg^{2+}) (Rathman, Sjaastad et al. 1996; Buchmeier, Blanc-Potard et al. 2000). In addition, neutrophils usually kill bacteria within phagosomes where superoxide and cytoplasmic constituents are contained. Myeloperoxidase is one such enzyme constituent, which converts chloride and hydrogen peroxide to hypochlorous acid (HOCl), which is strongly microbicidal (Winterbourn, Hampton et al. 2006).The macrophage intracellular environment is likely also to be at least microaerobic being bathed in the serum of oxygenated blood. Therefore, the assay was carried out aerobically. No difference between the growth patterns observed for the SL1344 single, double or triple nitrate reductase mutant when compared to the SL1344 wild type strain in MM5.8 medium was observed. This suggested that *Salmonella* has such a range of strategies to deal with the rigours of the macrophage attack that deletion of the known nitrate reductase systems does not compromise its survival under this set of challenging conditions.

Other work has shown that both *Pseudomonas aeruginosa and E. coli* suffer from motility and swarming defects when the activity of their Nar nitrate reductase or Hmp respectively is compromised (Schreiber, Krieger et al. 2007; Stevanin, Read et al. 2007). In motility assays carried out to assess any effect of this on

Salmonella, my results show that loss of the nitrate reductase systems in *S*. Typhimurium does not compromise motility.

To conclude, the NarGHJI of *Salmonella* mediates the assimilatory reduction of nitrate, as shown by the failure to thrive of the *ΔnarGHJI* deletion mutant in anaerobic GN medium.This is in agreement with work on *M. tuberculosis (Malm, Tiffert et al. 2009).* However, Nap is also able to compensate to some extent for the loss of NarGHJI in these conditions, since only growth of the *ΔnarGHJIΔnapDA* double deletion mutant accounted for the complete attenuation of growth observed in the triple mutant. This allies with work on *E. coli* (Potter, Millington et al. 1999; Mohan 2007)and in *M. tuberculosis* (Sohaskey 2008). This outcome was not improved by making another electron acceptor (fumarate) available. None of the deletions of nitrate reductases affected the motility of *Salmonella*, and this is at odds with the findings of previous work on *P. aeruginosa* (Schreiber, Krieger et al. 2007)and *E. coli* (Stevanin, Read et al. 2007). However, the triple mutant was shown to be more invasive in human epithelial cells than wild type, whether or not the SPI-1 TTSS had been induced prior to infection. This finding lead to *in vivo* work covered in the next chapter being carried out to see whether this phenomenon was carried through into a mouse model of infection. As has been alluded to already, the nitrate reductases are instrumental in the production of both NO and N_2O – perhaps this had some bearing on the invasiveness of *Salmonella*. Certainly, it is likely that the production of reactive nitrogen species both by the host and the nitrate reductases may be detrimental to other constituents of the microbiome (Henard 2011). Perhaps they also exert a damping effect on the progress of *Salmonella* into epithelial cells, which would otherwise result in a much more robust defence by the host and possible evacuation of the infective dose of *Salmonella* before a foothold could be gained*,* and a systemic infection initiated.

Chapter 5 – Characterization of nitrate reductase mutants *in vivo*

5.1 Introduction

If the role of the three nitrate reductase systems of *Salmonella*, NR-A, Nap and NR-Z, is to be fully elucidated, testing of all aspects of successive environmental adaptations of *Salmonella* which must take place in order for successful infection, colonization and invasion is vital. *S.* Typhimurium uses SPI-1 to invade epithelial cells (Shea *et al*., 1996) and Alston *et al* (unpublished data)have found that the SPI-1 genes are co-expressed with other genes of the *nap* operon (unpublished data) as raised above. Alston *et al* also found that a *nap* operon deletion mutant, tested for its ability to invade epithelial cells, showed a 20-30% attenuation of invasive capability compared with SL1344 wild type, which suggests that the *nap* operon could play a role in the early process of *Salmonella* infection. Since it is known, as described previously, that SPI-1 expression is a pre-requisite for the invasion process in *Salmonella*, this may suggest that the *nap* operon is also material to that process. The *nap* operon encodes one of the three nitrate reductase systems of *Salmonella*, Nap. It was therefore reasoned that the mutants to be tested for this project should include those components of the Nap operon involved in the construction of the periplasmic nitrate reductase. In addition, both of the other known nitrate reductase systems were deleted, comprising narGHJI and narZYWV. The possibility that there is a direct link between invasion and nitrate reduction is interesting, and invasion assays of epithelial cells provide a good starting point to explore any connection which exists.

5.2 Invasion Assays - Epithelial Cells

The invasion assays were carried out in human epithelial cells, HeLa, using a multiplicity of infection (MOI) of 1:1. The MOI relates the number of bacterial cells

to the number of epithelial cells which are present in an invasion assay, as a ratio. These assays therefore used an inoculum calculated to contain one bacterial cell for every epithelial cell present in the well. Higher MOIs may cause lysis of epithelial cells which would not normally occur *in vivo* (Maruyama *et al.*, 2007; Monack *et al.*, 2001). HeLa cells are an immortal human cell line which provide a well-defined and extensively tested model for the infection of mammalian cells using *S*. Typhimurium strains (Garcia-del Portillo, Zwick et al. 1993; Hardt, Chen et al. 1998; Steele-Mortimer, Meresse et al. 1999).

To ensure that an appropriate control was also used in these assays, a *Salmonella ΔrpoE* deletion mutant was also tested. Although this mutant has no direct involvement to this study, it is nevertheless relevant to use it as a control. It has been shown that a *ΔrpoE* mutant becomes challenged within the intracellular environment, and is less able to replicate within epithelial or macrophage cells than a comparable wild type strain (Humphreys, Stevenson et al. 1999; Cano, Martinez-Moya et al. 2001; Helaine, Thompson et al. 2010). RpoE is therefore vital to the virulence of *Salmonella spp.*

5.2.1 Results of invasion assays, in the absence of SPI-1 induction

Figure 27 shows the ability of the bacteria to invade the epithelial cell monolayer initially, in this case, even without prior induction of the SPI-1 type three secretion system. Each of the double nitrate reductase mutants vary little from the wild type in their invasive capability; however, the triple mutant is over one third more invasive than the wild type. The ability of the bacteria to replicate within the epithelial cells once they have gained ingress, which is achieved through the deployment of the SPI-1 type three secretion system (TTSS), is illustrated in Figure 28.The baseline measurement was assessed after two hours following the

initial infection and it is generally accepted that the number of internalized bacteria at this timepoint accurately reflects the numbers of bacteria which invade from the inoculum, and before any measurable replication of bacteria has taken place (Finlay, Heffron et al. 1989; Gahring, Heffron et al. 1990; Kusters, Mulders-Kremers et al. 1993).Following internalization, the bacteria need to equilibrate before they switch to a fully replicative phenotype.This follows the acidification and shrinking of the phagosome to fit around the internalized bacterium to form the SCV. Subsequently, induction of the SPI-2 TTSS within the SCV results in translocation of effector proteins into the SCV several hours after the initial internalization, and which allow replication of the bacteria to ensue (Haraga, Ohlson et al. 2008).

Six hours following the infection, the epithelial cells were lysed and their bacterial contents released and enumerated; this confirmed that all the strains tested were able to replicate within the HeLa cells, and hence develop increased bacterial numbers over time, following the initial inoculation of the monolayer with bacteria, compared to the bacterial numbers present within the epithelial cells two hours following infection.

Figure 28 summarisesthe fold change which was measured in bacterial numbers of SL1344 wild type and double and triple nitrate reductase and rpoE deletion mutants, within HeLa human epithelial cells given an extra four hours following the infection. All the strains tested replicated within the HeLa cells, although the *ΔrpoE* deletion mutant, used as a control, was the least able to increase its bacterial numbers. This was expected, since as explained earlier, replication of this mutant is known to be inhibited intracellularly. Overall, there was no difference between the replicative ability of any of the nitrate reductase mutants compared to the SL1344 wild type strain.

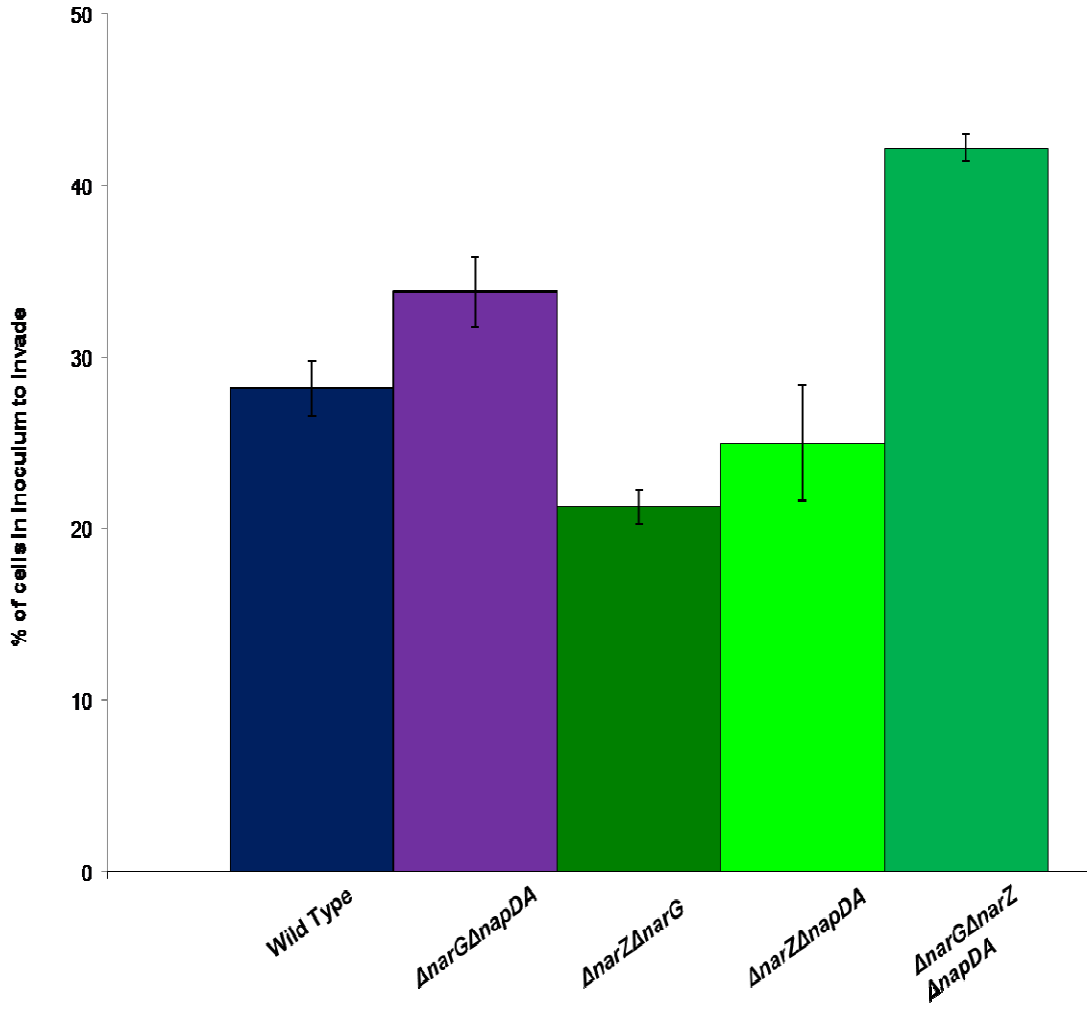


Figure 27 – Invasion levels in SL1344 wild type and double and triple nitrate reductase deletion mutants in HeLa human epithelial cells without SPI-1 induction. The results have been adjusted to account for variations in the initial inoculum applied to the epithelial cell monolayer. Error bars show the Standard Error of the Mean for three separate experiments.

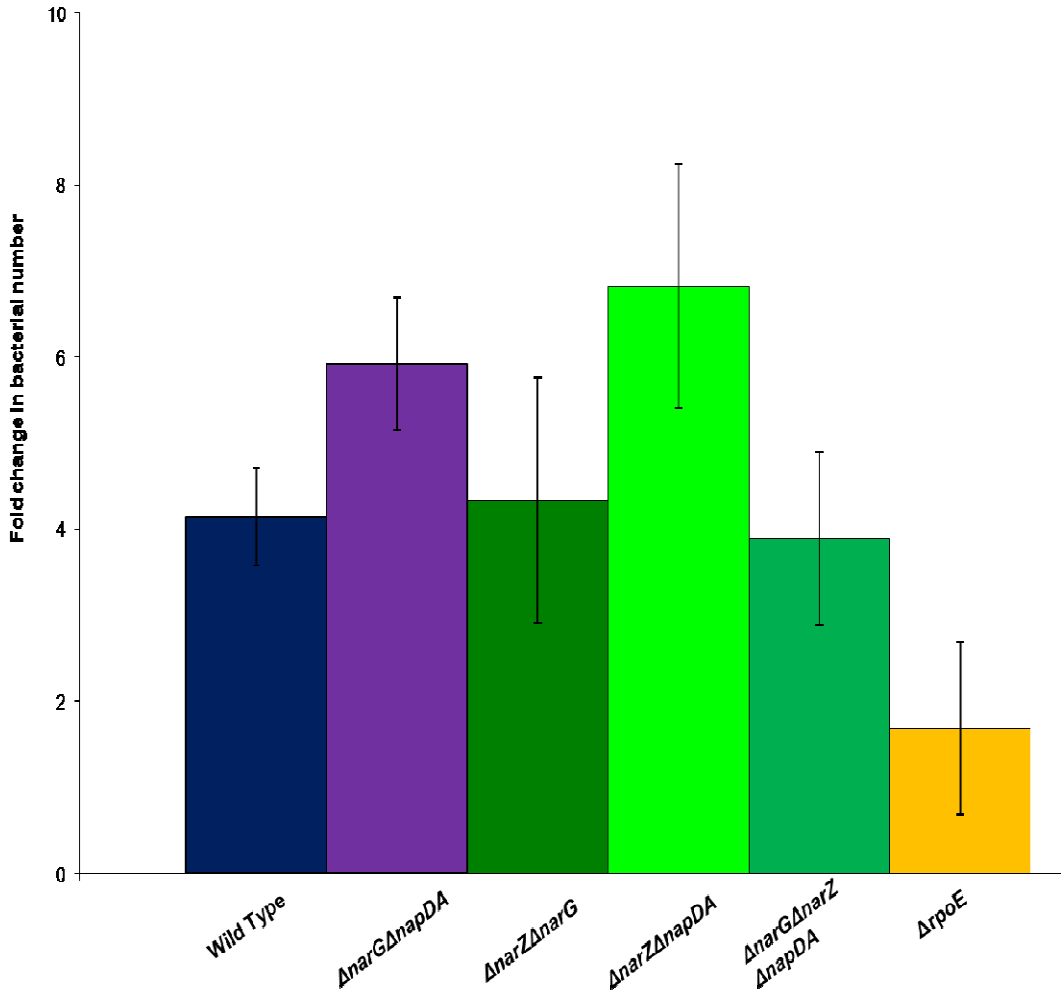


Figure 28 – The fold change in bacterial numbers of SL1344 wild type and double and triple nitrate reductase and rpoE deletion mutants, within HeLa human epithelial cells in four hours. The ΔrpoE deletion mutant was used as a control. Error bars show the Standard Error of the Mean for three separate experiments.

5.2.2 Results of invasion assays of HeLa cells with SPI-1 induction

As was discussed earlier, entry of *Salmonella* into epithelial type cells of the gastrointestinal tract is enhanced through the use of the SPI-1 TTSS apparatus. Consequently, it was decided to carry out further invasion assays to test the nitrate reductase mutant strains and the ΔrpoE mutant, compared to the SL1344 Wild type strain, having first induced the expression of the SPI-1 system by subculturing overnight cultures of the bacteria in LB-S, an accepted method to induce SPI-1 production (Ibarra, Knodler et al. 2010; Garcia-Calderon, Casadesus et al. 2007; Temme, Salis et al. 2008).

In Figure 29, each of the double nitrate reductase mutants vary little from the wild type in their invasive capability. However, the triple mutant is a little less than one third more invasive than the wild type, and this reflects the result shown in Figure 27 which was carried out without prior SPI-1 induction.

The difference in bacterial numbers of SL1344 wild type and double and triple nitrate reductase and rpoE deletion mutants, within HeLa human epithelial cells over time is shown in Figure 30. All the nitrate reductase deletion strains tested replicated and increased their bacterial numbers within the HeLa cells over time; consequently, they show increased bacterial numbers six hours after the initial inoculation of the monolayer with bacteria, compared to the bacterial numbers present within the epithelial cells two hours following infection. However, the *ΔrpoE* deletion mutant exhibited a reduction in bacterial numbers compared to the two hour timepoint, suggesting that not only was this mutant unable to replicate normally, it may have even been dying off. This finding is supported by other work (Cano, Martinez-Moya et al. 2001), in which lack of the RpoE alternative sigma factor caused considerable reduction in intracellular viability of *S*. Typhimurium.

Figure 30 alsoshows the fold change in bacterial numbers of SL1344 wild type and double and triple nitrate reductase and *ΔrpoE* deletion mutants, within HeLa human epithelial cells in four hours. All the nitrate reductase deletion mutant strains had replicated within the HeLa cells. In the case of the triple deletion mutant, replication levels were about 65% higher than in the wild type strain. However, in the case of the *ΔrpoE* deletion mutant, used as a control, the bacterial numbers dropped over time, as indicated in Figure 30. Overall, there was no difference between the replicative ability of any of the double nitrate reductase mutants compared to the SL1344 wild type strain.

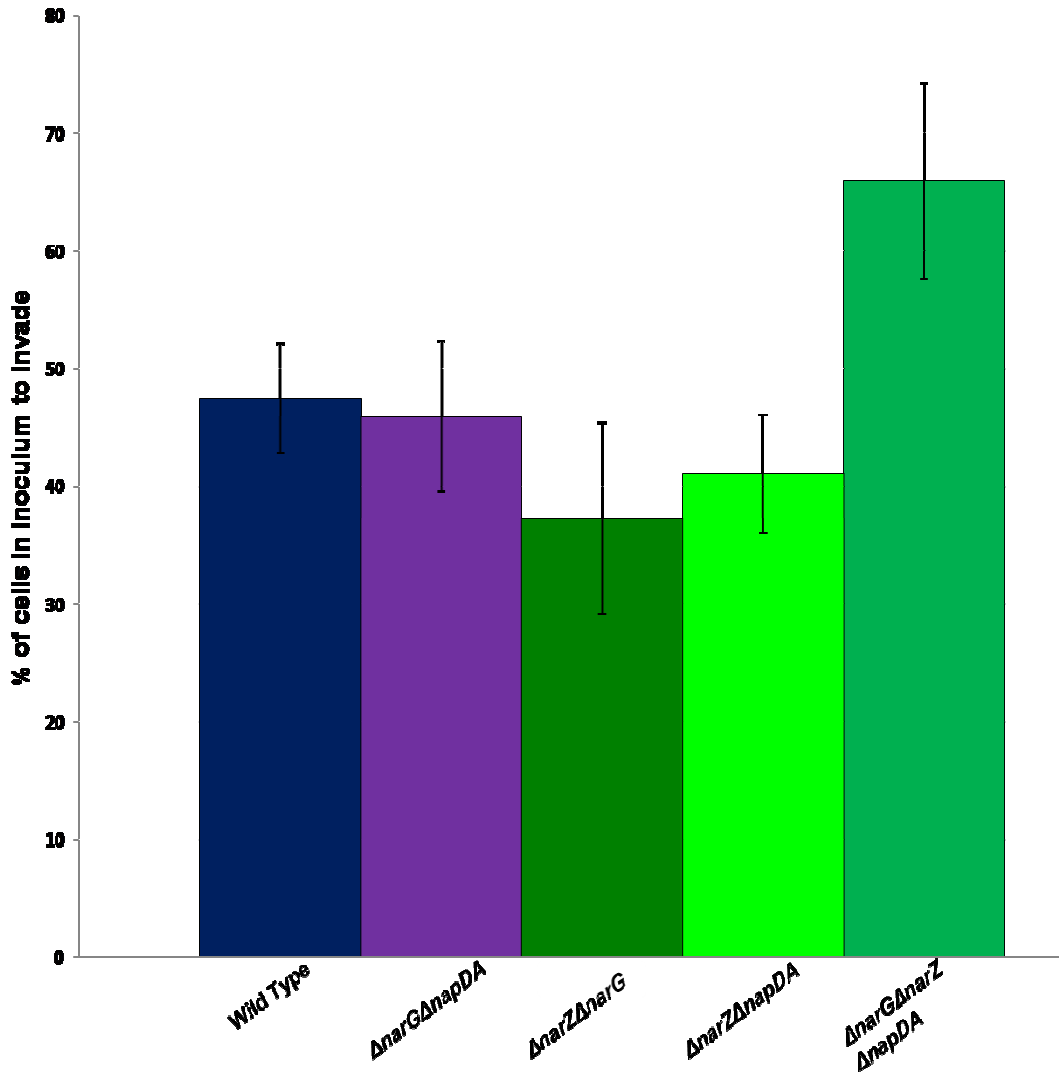


Figure 29 - Invasion levels in SL1344 wild type and double and triple nitrate reductase deletion mutants, in HeLa human epithelial cells with SPI-1 induction. The results have been adjusted to account for variations in the initial inoculum applied to the epithelial cell monolayer. Error bars show the Standard Error of the Mean for three separate experiments.

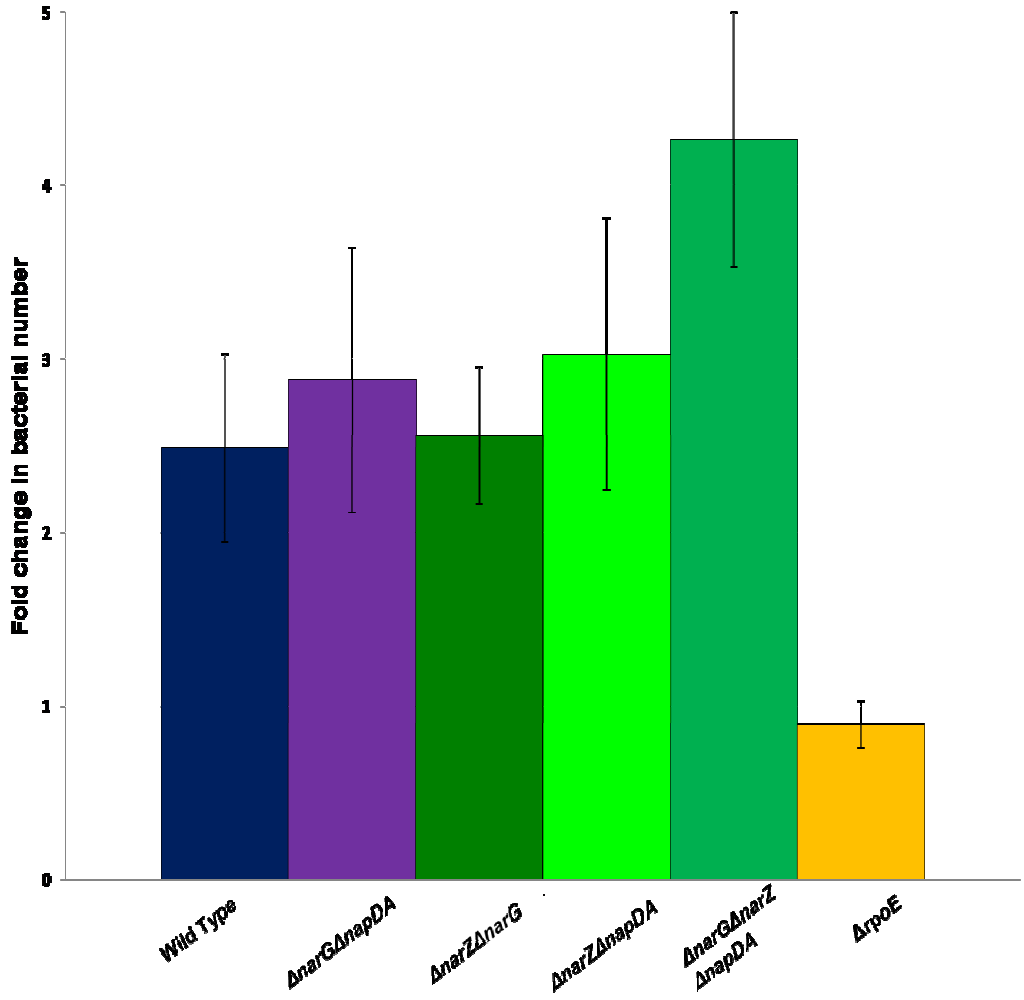


Figure 30 – The fold change in bacterial numbers of SL1344 wild type and double and triple nitrate reductase and *ΔrpoE* deletion mutants, within HeLa human epithelial cells in four hours. The ΔrpoE deletion mutant was used as a control. Error bars show the Standard Error of the Mean for three separate experiments.

5.2.3. Gentamycin Protection Assays in RAW264.7 Murine Macrophages

Unlike the requirement for an operative SPI-1 apparatus to enhance ingress into the cells, the situation with macrophage cells is different to that which occurs in epithelial cells and which has been alluded to in Section 5.3.2 above. Since macrophages are phagocytic in nature, there is no need for the bacteria to manipulate the cellular cytoskeleton to gain access, since the role of the macrophage is to envelope and ingest the bacterium, with a view to destroying it. In consequence, it is not necessary to induce SPI-1 production in assays using macrophage cells. However, in most other respects, the protocol for macrophage protection assays is broadly similar to that used for the epithelial cell invasion assays carried out previously.

Figure 31 illustrates the fold change in bacterial numbers of SL1344 wild type and nitrate reductase single operon deletion mutants, within RAW264.7 murine macrophages between 2 and 24 hours post-infection. All the nitrate reductase deletion mutant strains replicated within the macrophages, and overall, there was no difference between the replicative ability of any of the nitrate reductase mutants compared to the SL1344 wild type strain.

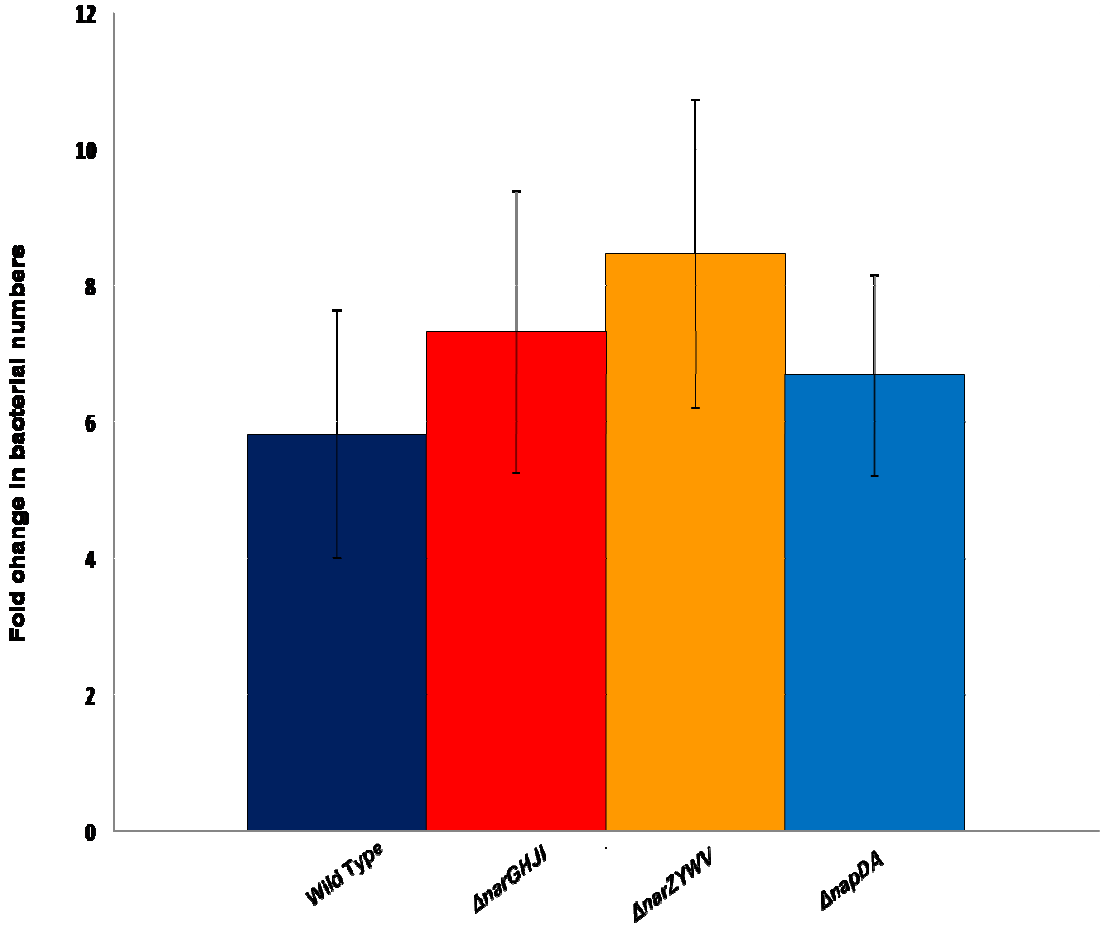


Figure 31 - The fold change in bacterial numbers of SL1344 wild type and nitrate reductase single operon deletion mutants, within RAW264.7 murine macrophages between 2 and 24 hours. Error bars show the Standard Error of the Mean for three separate experiments.

5.3 Infection of Balb/C mice

5.3.1 Introduction

Having exposed what potentially appeared to be a hyperinvasive phenotype in the triple nitrate reductase deletion mutant *ΔnarGHJIΔnarZYWVΔnapDA*, it was decided that further testing of this possibility was justified, by using an *in vivo* model. The activity of the nitrate reductase system has been linked to virulence for other bacterial species which are also pathogens of humans, for example *M. tuberculosis* (Weber, Fritz et al. 2000) and *P. aeruginosa* (Palmer, Brown et al. 2007; Van Alst, Picardo et al. 2007). The observation we made of possible hyperinvasiveness using *Salmonella* had been made during the invasion of epithelial cells, but not entry to macrophages. A major difference between the process of invasion of epithelial cells, and ingress into macrophages, is that to enter epithelial cells, *Salmonella* employs the products of the SPI-1 TTSS, which subverts normal host cell activity so that the epithelial cells behave more like phagocytic cells and ingest the bacteria. This facility is not required to gain entry to macrophages, which are naturally phagocytic, and seek out and ingest foreign particles, including bacteria, without the need for bacteria to cajole them into doing so. Since the only way *in vivo* in which bacteria would have to gain entry through the employment of the SPI-1 machinery would be via the oral route of infection, experiments were conducted using this method in Balb/C mice, to further test this phenomenon.

5.3.2 Oral Infection Assays

Population levels of SL1344 wild type bacteria were compared to the bacterial numbers of the SL1344*ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase

deletion mutant, following oral infection of Balb/C mice. During the early stages of infection, in the stomach, intestine and colon, there was little to choose between the wild type and mutant levels of viable bacteria present. However, once the infection had progressed to the systemic phase, as demonstrated in the caecum, Peyer's patches, spleen and liver, the mutant demonstrated increased numbers of bacteria, and hence virulence, compared to the the wild type strain. This finding is illustrated in Figure 32.

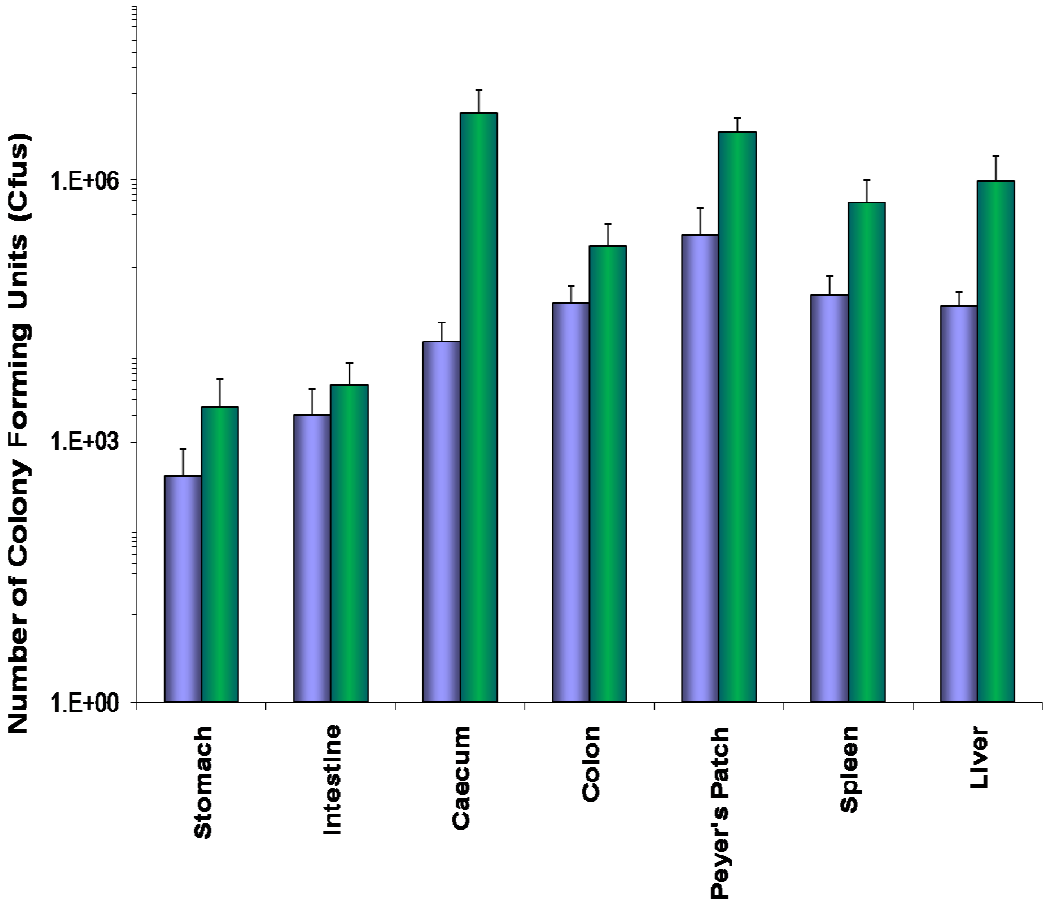


Figure 32 Population levels of SL1344 wild type compared to SL1344*ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutant, following oral infection of Balb/C mice. Wild type (blue bars)Triple mutant (green bars). Error bars show the Standard Error of the Mean for three separate experiments.

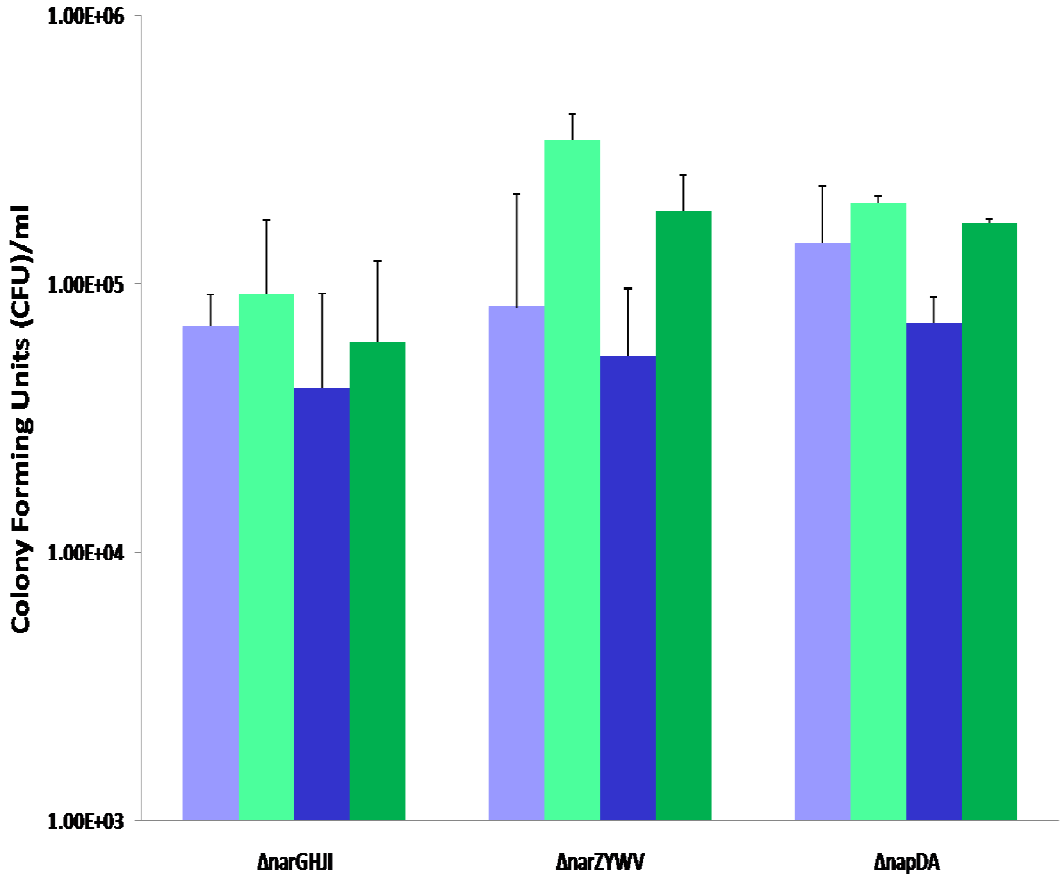
5.3.3 Intraperitoneal Infection Assays

5.3.3.1 Competitive Index experiments

Previously, the LD_{50} method, in which the number of bacteria required to kill half of the infected model animals is critical, has been widely used as an indicator of virulence or of attenuation. However, since this also shows the collective effects of lethality, (i.e. not all mouse death may be due to the effects of the bacterial inoculation or infection), it is rather insensitive, and it is not possible to use it to show where in the infection process the gene of interest may be deployed (Beuzon and Holden 2001). Scoring of the symptoms of infection displayed by the infected animals, although part of the experimental protocol, is used to ensure that there is no unnecessary suffering of the animals. It is too subjective a measure, being based in the judgement exercised by the practitioner, for firm conclusions to be drawn from it.

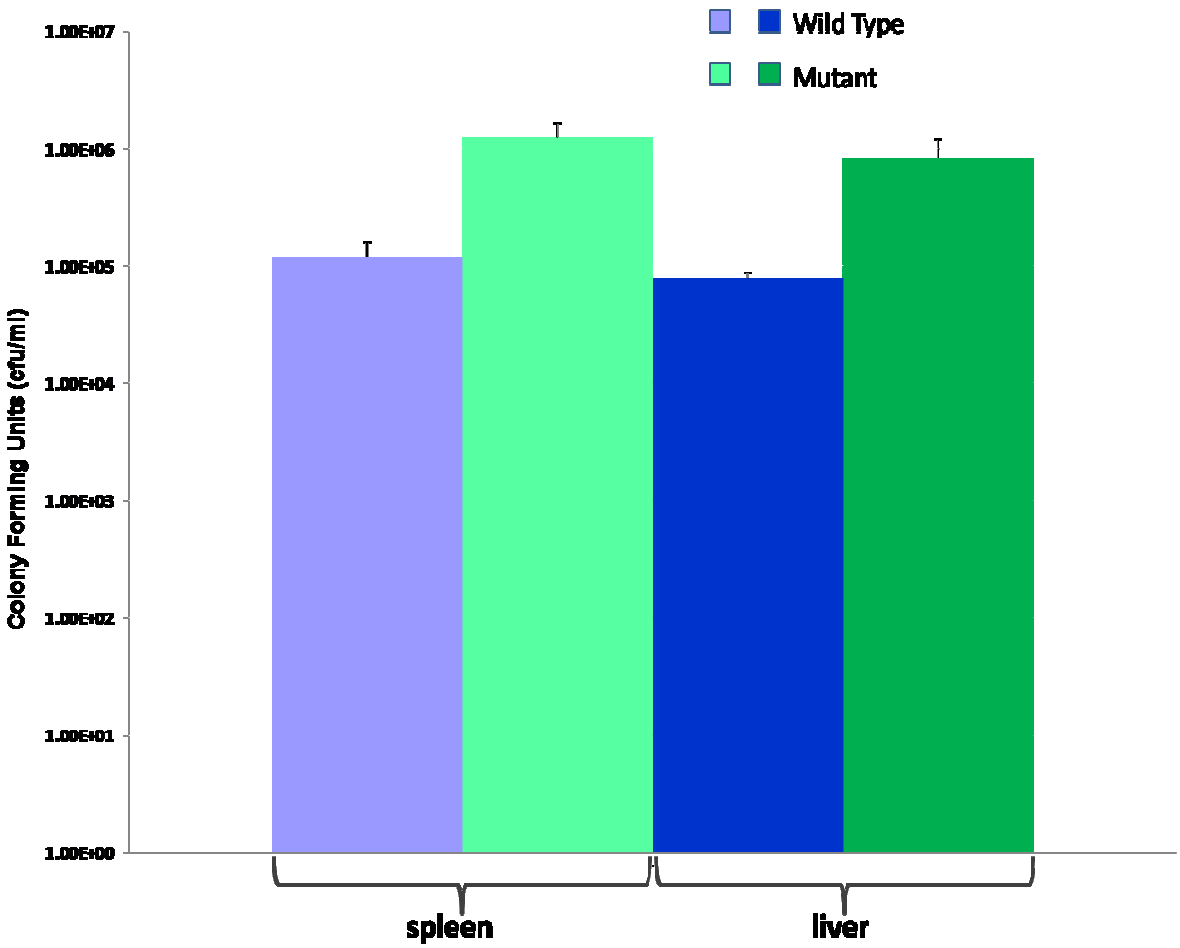
Use of the Competition Index (CI) however, provides a more robust illustration of virulence since it reflects bacterial numbers instead of mouse survival rates (Beuzon and Holden 2001). The CI method requires the administration of a combined inoculum containing equal numbers of both wild type and mutant strains. Administration of the inoculum is achieved via an intra-peritoneal injection. After a suitable time interval, the animals are sacrificed and the bacteria recovered from particular organs (liver and spleen) which are known to harbour the infective bacteria post-infection. Viable counts of these bacteria can then be enumerated, and identified by selective screening using their antibiotic susceptibility. The CI calculation illustrates the relative virulence ratio (RVR) between two bacteria strains. It compares the ratio of the mutant strain to the wild type strain in the output number of recovered bacteria, divided by the ratio of the two strains to each other in the original inoculum (Monk, Casey et al. 2008). A value of 1 indicates no change in the RVR.

Both the spleen and liver of infected animals contained more bacteria of each of the mutant strains than the wild type strain, using the single nitrate reductase mutants, as shown in Figure 33. When animals were infected intraperitoneally with the triple nitrate reductase deletion mutant, SL1344*ΔnarGHJIΔnarZYWVΔnapDA*, the same trend was maintained and the numbers of the mutants which could be isolated from both tissue types, liver and spleen, were higher for the mutant than for the wild type strain, illustrated in Figure 34. However, the results of the CI calculations made suggest that the RVR is no different between the wild type and any of the mutant strains tested.



Spleen RVR	**0.89**	**1.58**	**1.77**
Liver RVR	**0.81**	**0.17**	**0.62**

Figure 33 - Competitive Index comparison of SL1344 wild type and SL1344 *ΔnarGHJI*, *ΔnarZYWV*, and *ΔnapDA* single operon deletion mutants.Bacterial numbersin the spleen are shown for wild type (pale blue bars) and each single nitrate reductase deletion mutant (pale green bars). In the liver, bacterial numbers are illustrated for wild type (dark blue bars) and for each single mutant (dark green bars). Error bars show the Standard Error of the Mean for three separate experiments.



Spleen RVR 0.97 **Liver RVR 0.94**

Figure 34 - Competitive Index comparison of SL1344 wild type and SL1344 *ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutant. Bacterial numbers in the spleen and liver are shown for wild type and the triple nitrate reductase deletion mutant (dark green bar). Error bars show the Standard Error of the Mean for three separate experiments.

5.4 Discussion

S. Typhimurium uses SPI-1 to invade epithelial cells, (Shea *et al*., 1996) and Alston *et al.* have found that the SPI-1 genes are co-expressed with other genes of the *nap* operon (unpublished data) as detailed previously. Alston *et al* previously tested the ability of a *nap* operon deletion mutant to invade epithelial cells, and found an apparent 20-30% attenuation of invasive capability compared with SL1344 wild type (Alston *et al*., unpublished data). This suggests that the *nap* operon plays a role in the early processes of *Salmonella* infection. Since the *nap* operon encodes one of the three nitrate reductase systems of *Salmonella*, Nap, this suggested a direct link between invasion and nitrate reduction. Invasion assays of epithelial cells provided a good starting point to explore any connection, and were carried out using human epithelial cells, (HeLa), using a multiplicity of infection (MOI) of 1:1. This was intended to be physiologically relevant; higher MOIs may cause lysis of epithelial cells which would not normally occur *in vivo* (Maruyama *et al.*, 2007; Monack *et al.*, 2001).

The control used, a *Salmonella ΔrpoE* deletion mutant, has no direct link to this study, but has been previously shown to be less able to replicate within epithelial or macrophage cells than a comparable wild type strain (Humphreys, Stevenson et al. 1999; Cano, Martinez-Moya et al. 2001; Helaine, Thompson et al. 2010). RpoE is consequently of use here for comparison purposes.

Experiments were conducted with and without priming of the SPI-1 TTSS by induction. *Salmonella* switches on this machinery in response to environmental cues in readiness for invasion of epithelial cells *in vivo*. It was found that while the double mutants varied little from the wild type in terms of their invasive ability, the triple mutant was about two-thirds more invasive. However, once inside the epithelial cells, it was shown that there was no significant difference between the

replicative ability of any of the nitrate reductase mutants compared to the SL1344 wild type strain.

As was discussed earlier, entry of *Salmonella* into epithelial type cells of the gastrointestinal tract is achieved through the use of the SPI-1 TTSS apparatus. Consequently, further invasion assays to test the strains in this study were carried out having first induced the production of the SPI-1 system. Once again, it was found that the triple mutant was around one-third more invasive than the wild type, while the double mutants varied very little from the wild type invasion levels. Also, replication of the bacteria once inside the epithelial cells was maintained at a level commensurate with that in the wild type for all the mutant strains.

In macrophages, there is no requirement for an operative SPI-1 apparatus to enhance ingress into the cells, since the phagocytic nature of the macrophage means that it will actively seek to engulf the bacteria. No difference between numbers of wild type and mutant bacteria which gained ingress to macrophages was observed. There was also no significant difference in the replicative ability of the mutants compared to wild type once inside macrophages.

Population levels of SL1344 wild type bacteria were compared to the bacterial numbers of the SL1344*ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutant, following oral infection of Balb/C mice. In the initial stages of the infection, during the passage and occupation of the murine stomach, intestine and colon by the bacteria, there was little difference between the numbers of the mutant strain bacteria which were isolated and the wild type strain. However, following advancement of the infection to the systemic phase, which follows invasion and sequestration of the bacteria within macrophages, as demonstrated by the numbers of viable bacterai isolated from the caecum, Peyer's patches, spleen and liver, the mutant demonstrated increased virulence over the wild type. The removal of all three nitrate reductase systems may have also reduced the levels of NO and N_2O which are normally produced endogenously by these

systems. This would in turn have reduced the environmental challenge to these bacteria, so that more were able to expend energy in survival and replicative functions, increasing their numbers present in the systemic samples.

Interpretation of these data lead to the conclusion that nitrate reductase mutants, of the Nar and Nap operon systems, are not compromised in their ability to employ the SPI-1 TTSS to invade epithelial cells of the mammalian gastrointestinal tract. However, once they have gained access, they may well be able to replicate more efficiently within the macrophages of their host, so increasing the bacterial load within tissues and hence the virulence of the infection. This was also evidenced by the (admittedly slightly subjective) observations made of the condition of the animals during the experiments. All the animals, regardless of the strain with which they had been inoculated required to be sacrificed by day 5, due to their poor condition and to prevent unnecessary suffering. However, it was observed and noted that those inoculated with the mutant strain appeared to be slightly more unwell in appearance than those which had been given the wild type strain. It was also observed that the changes in the mice with the mutant infection occurred more dramatically in the final day and hours of the experiment, lending weight to the possibility that having gained a higher critical mass of bacterial load, due to an increased propensity for replication, the effects snowballed and would result in a swifter demise had the infection been permitted to run its natural course.

SPI-1 mutants are still able to infect the host following intraperitoneal (IP) infection to the same extent as wild type *Salmonella* (Galan 1996; Ochman, Soncini *et al*. 1996). Further, SPI-1 is not necessary for intracellular survival and the systemic phase of any infection. However, SPI-1 mutants fail to infect the host following oral infection, and also are not able to invade enterocytes *in vitro* (Lucas and Lee 2001). This supports the premise that SPI-1 is required for invasion via the gut epithelium. Since I found that nitrate reductase mutants are

still able to invade via the gut epithelium, it is possible to extrapolate that loss of the nitrate reductase systems does not also compromise expression of the components required for production and assembly of the SPI-1 TTSS. Consequently, I conclude that despite the findings of Alston *et al* that genes of the *nap* operon are coexpressed with those of the SPI-1 TTSS, it is not because they are required for the efficient deployment of SPI-1.

It seems that the nitrate reductase systems do, however, ameliorate the replicative capacity of *Salmonella* within macrophages, since loss of the systems causes an increase bacterial replication. The mechanisms which cause a reduction of replication are worthy of further investigation, since it might be possible to further exploit them in the design of effective treatments for the illnesses caused by *Salmonella* infections. I would propose that the reduction in the endogenous NO production may provide an interesting place to start. The nitrate reductase systems appear to place a brake on the ability of *Salmonella* to overwhelm the host defences. This may help to ensure that the infection lasts long enough to ensure that it is passed on before the host is destroyed. Several pathogens are known to control their potential for destruction of their host to ensure their own survival. Consideration of the evolutionary advantage which is or may be available to organisms which control their virulence leads to the conclusion that there is a close relationship between virulence and the effect on host mortality (Lenski and May 1994; Koch 2007)

In this regard, other previous work has shown also that the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant was not attenuated after intraperitoneal infection of mice, or in cultured murine macrophages (G. Rowley, unpublished data). This is a mutant which should experience a reduced capacity to detoxify NO, while the nitrate reductase triple deletion mutant constructed in this study may also produce less NO than the wild type. Since neither of these mutants are attenuated systemically, perhaps there are either other mechanisms which are

working to equilibrate any effect of NO on *Salmonella,* which have yet to be fully explored, or NO is simply not important against *Salmonella* once it is protected within the SCV of mammalian macrophages.

In summary, these data suggest that what has been identified in the course of this work is a highly replicative phenotype in the nitrate reductase mutants of *Salmonella* Typhimurium rather than a phenotype which is capable of highly increased invasion levels when ingested via the oral route.

Chapter 6 – Regulation of Nitrate Reduction Systems

6.1 Introduction

As has already been described, the SPI-1 expression is a vital component of the infection process for *Salmonella*, and a major regulator of SPI-1 expression is HilA (Thompson, Rowley et al. 2006). Fis is also involved in SPI-1 expression (Wilson, Libby et al. 2001), in that it is required for the expression of HilA (Wilson, Libby et al. 2001; Schechter, Jain et al. 2003). Fis activity has also been implicated in the virulence of *Shigella* and enteroinvasive strains of *E. coli* (EIEC) (Falconi, Prosseda et al. 2001) through its interaction with the DNA binding protein H-NS. The ferric uptake regulator, Fur, also positively regulates HilA by the repression of H-NS (Troxell, Sikes et al. 2010). FNR is another DNA binding protein which has been described as a global regulator of *Salmonella* virulence, driven by oxygen sensitivity (Fink, Evans et al. 2007). CRP is a transcription regulator related to FNR, and also involved in DNA-binding (Spiro, Gaston et al. 1990), while the alternative sigma factor RpoS has long been recognised to regulate virulence in *Salmonella* (Fang, Libby et al. 1992). While FNR and CRP have also been investigated extensively in *E. coli*, HilA is exclusive to *Salmonella* since it is linked to expression of the SPI-1 TTSS, machinery which is not employed by *E. coli*.

To ascertain the extent, if any, of the link between regulation of *Salmonella* virulence and the *nap* operon, which had been hinted at by the work of Alston *et al* (unpublished), β-Galactosidase assays were carried out using mutants in each of these systems, in a *Salmonella* Typhimurium background strain for comparison purposes.

6.2 β-Galactosidase Assays

B-Galactosidase is encoded by the *lacZ* gene of the *lac* operon in *E. coli*. It is a large (120 kDa, >1000 amino acids) protein that forms a tetramer. The enzyme cleaves lactose to glucose and galactose by hydrolysis within the cell so that they can be used as carbon/energy sources. The synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) is also recognized as a substrate and cleaved to yield galactose and o-nitrophenol which has a yellow colour. When ONPG is in excess over the enzyme in a reaction, the production of o-nitrophenol per unit time is proportional to the concentration of β-Galactosidase; thus, the production of yellow colour can be used to determine enzyme concentration.

6.3 Results

Figures 35, 36 and 37 inclusive illustrate the results of β-Galactosidase Assays carried out under aerobic conditions, and 38 and 39 are concerned with the results of anaerobic assays.

Figure 35 shows that the transcription factor FNR (fumarate nitrate reduction) and the alternative sigma factor RpoS both exert positive regulation over NapF, under aerobic conditions. The greatest influence is exerted by FNR which drives an almost three-fold increase in expression aerobically compared to the wild type. The alternative sigma factor RpoS on the other hand doubles the aerobic expression compared to wild type. Negative regulation is shown by both Fis and Crp, which each reduce expression by around one half. However, it is noticeable that the actual number of Miller Units measured is quite small, so the differences between them have to be viewed with an element of caution, and their significance should not be overestimated.

It is interesting that under anaerobic conditions, RpoS undergoes a change in its activity; RpoS switches from positive regulation aerobically, to negative regulation in the absence of oxygen, and this is exquisitely illustrated in Figure 38. Significantly, here it demonstrates almost 100-fold inhibition of expression compared to the wild type, which is also to the same level as that exhibited by both HilA and Crp. Further, it is of note that the Miller Units measured in this assay are much higher, so differences are also much greater, and hence, more meaningful. The other negative regulators of NapF under anaerobic conditions, confirmed by the results, HilA and Crp, also demonstrate robust negative regulation anaerobically, along with, to a lesser extent, Fis. The result for Fis, however, is marginal. In the meantime, Fnr exerts the opposite effect of positive regulation of NapF in anaerobic conditions; indeed it would curtail expression of NapF in its entirety.

In the case of NarG, Figure 36 shows that Fis exerts a high level of negative control under aerobic conditions, on *S*. Typhimurium, which equates to almost a four hundred-fold inhibition. In Figure 37, in which the data for the *Δfis* variant has been removed for purposes of clarity, it is possible to see that Crp also provides a (much lower) level of negative control over NarG, while HilA, FNR, and RpoS all regulate NarG positively, but at low levels. However, the Miller Units calculated here are small numbers, so differences, although replicated in this work, should be afforded some caution. Further, reference to Figure 39 indicates that anaerobically in *S*. Typhimurium, Fis exerts a positive regulatory role on NarG, the opposite of what was observed under aerobic conditions. Like the effects of RpoS on NapF expression, therefore, Fis adopts opposing influences under differing conditions of aerobiosis. HilA is the only negative regulator of NarG in the absence of oxygen; all the other regulators which were tested displayed positive regulation on NarG in these conditions. Anaerobically, HilA demonstrates

100-fold enhancement of NarG expression, while Crp and Fis exert negative influences of around 10-fold.

In summary, my results indicate that NapF undergoes negative regulation by four of the six regulators tested anaerobically, which also includes HilA. NarG is negatively regulated only by HilA under the same anaerobic conditions. Conversely, in aerobic conditions, both NapF and NarG are negatively regulated by the same two of the tested regulators, Crp and Fis.

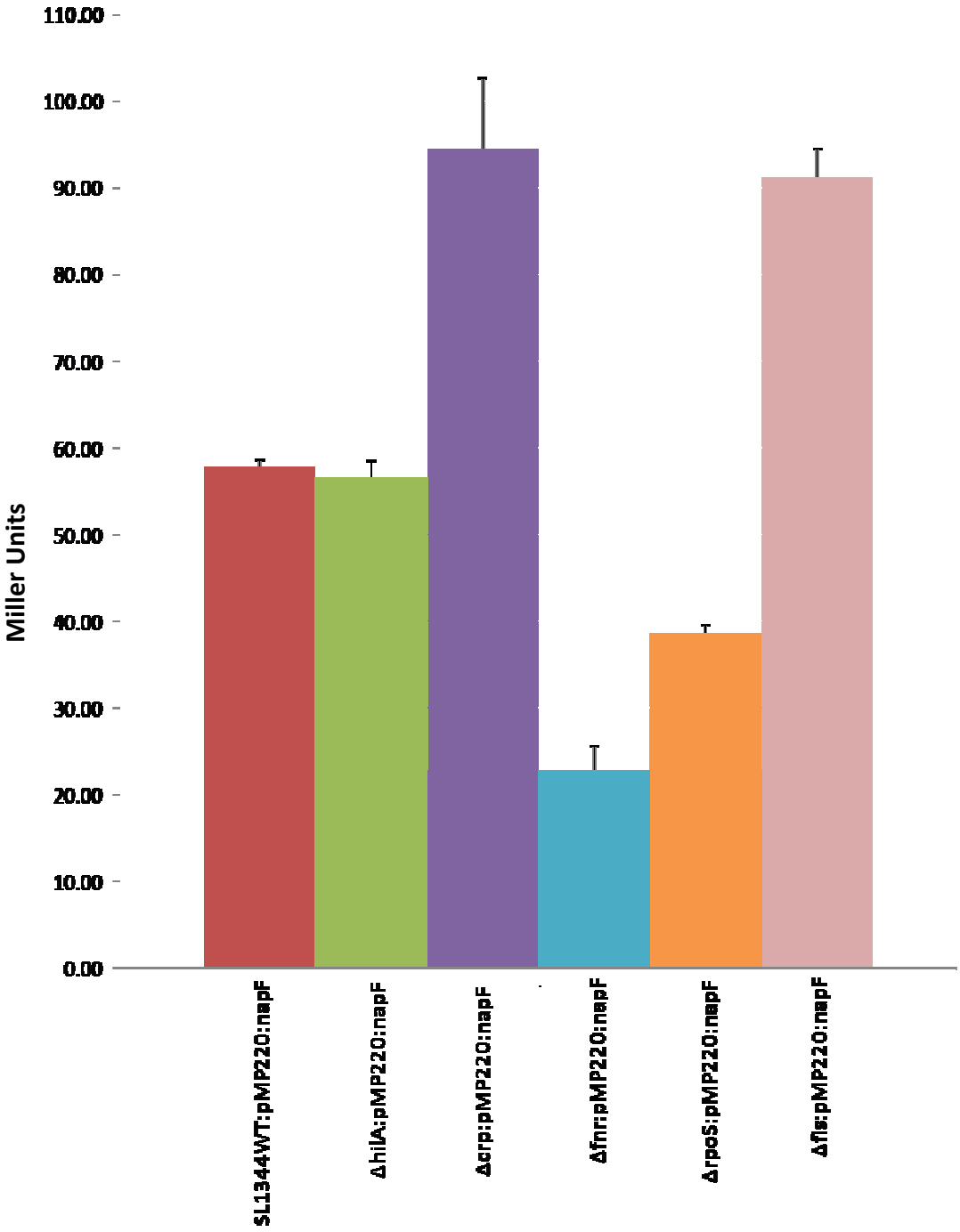


Figure 35 - Aerobic β-Galactosidase assay pMP220 with napF insert, excluding the empty plasmid. Results suggest that FNR and RpoS exert positive regulation over NapF (■ and ■). Conversely, Crp and Fis demonstrate negative regulation of NapF (■and ■). Miller units for NapF in Fnr, Fis and rpoS background significantly different (student t test, $p<0.05$) to WT. Error bars show the Standard Error of the Mean.

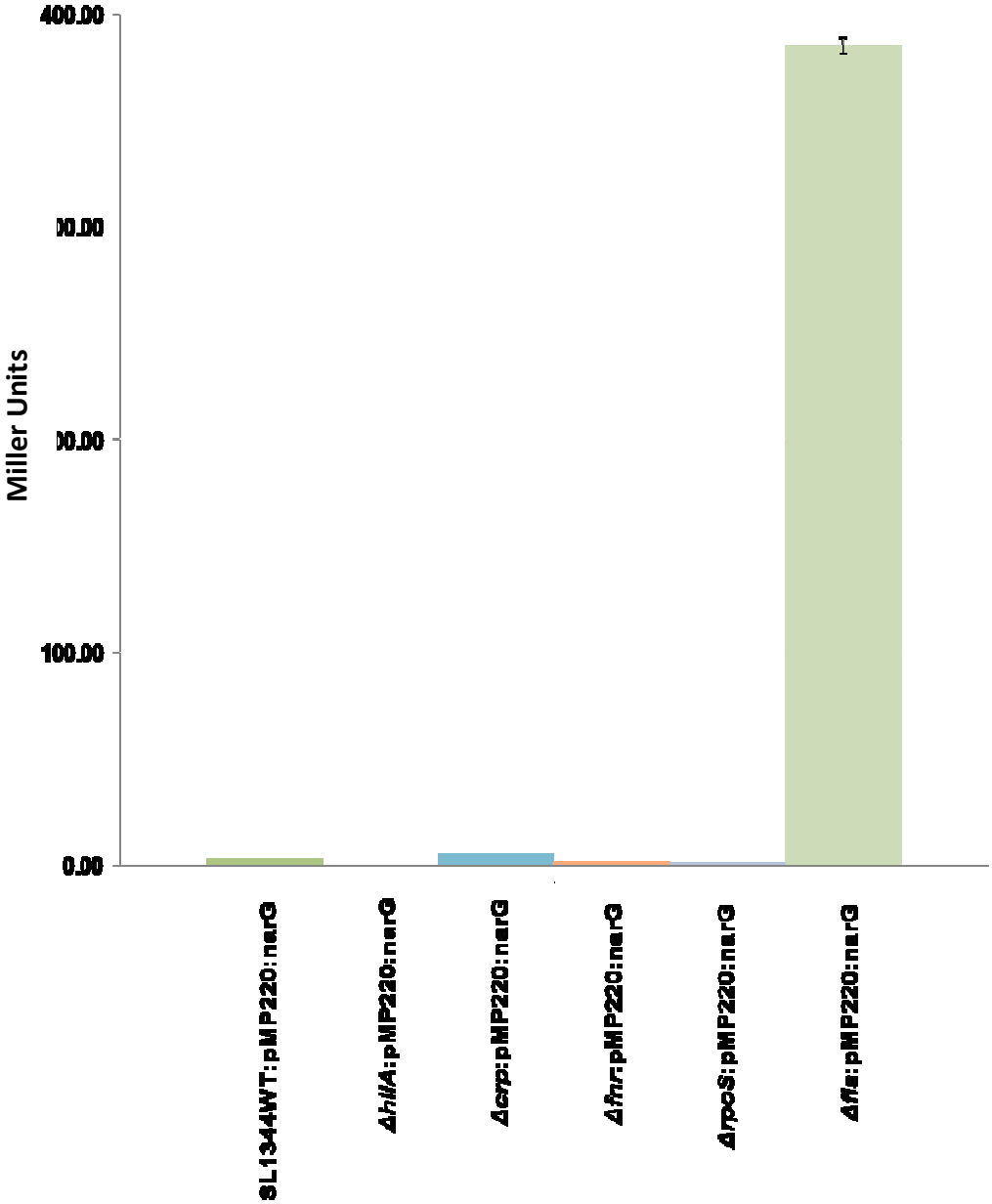


Figure 36 - Aerobic β-Galactosidase assay with pMP220 with narG insert, excluding the empty plasmid. Results suggest that Fis (■) exerts a high level of negative control over NarG. Figure 37 focusses more accurately on the results for the other variants included in this assay, by removing the data for *Δfis* variant.Miller units for NarG in Fis background significantly different (student t test, $p<0.05$) to WT. Error bars show the Standard Error of the Mean.

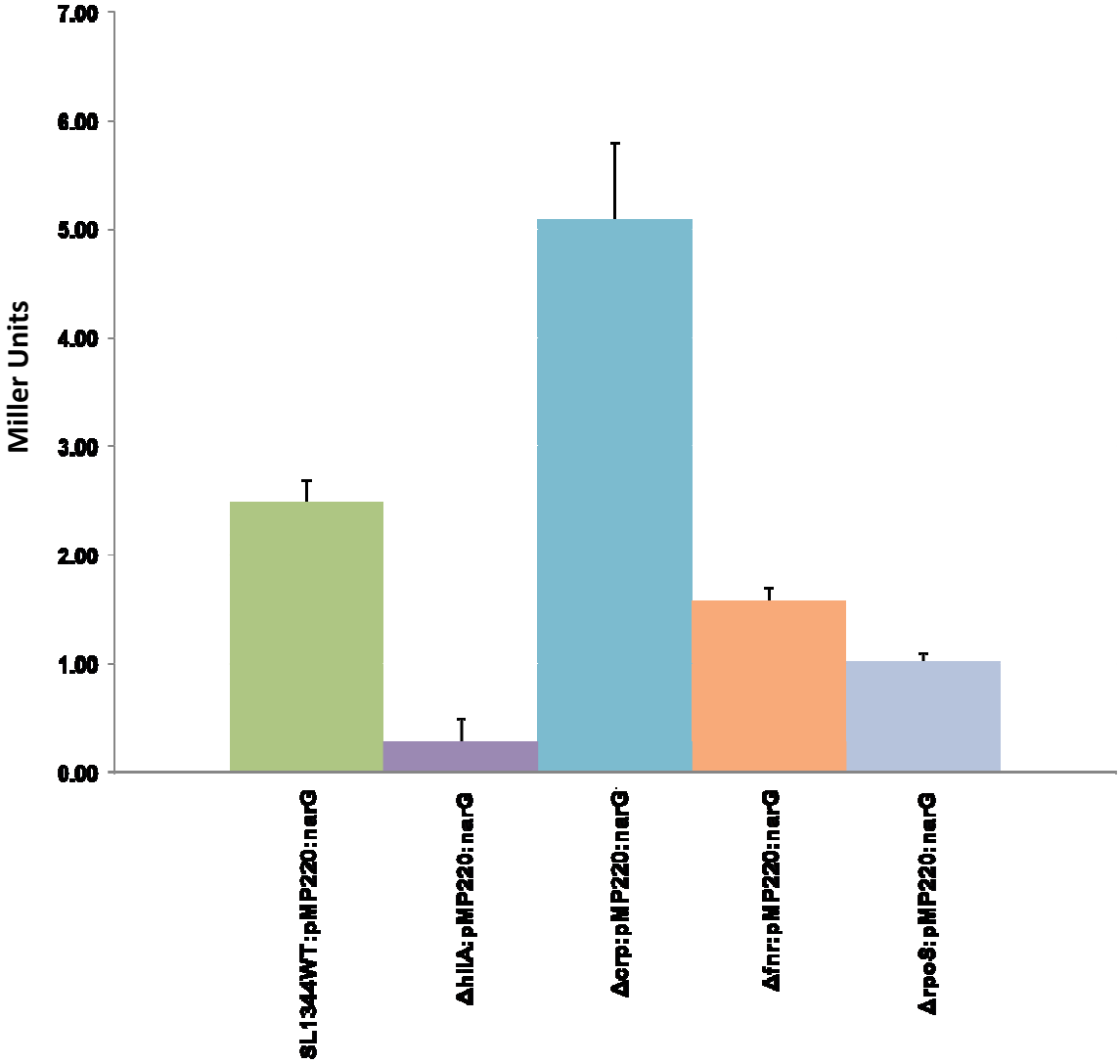


Figure 37 - Aerobic β-Galactosidase Assay with narG insert, excluding empty plasmid and excluding *Δfis*. Results suggest positive regulation by HilA (■), Fnr (■) and RpoS (■)and negative regulation in the case of Crp (■). Error bars show the Standard Error of the Mean.

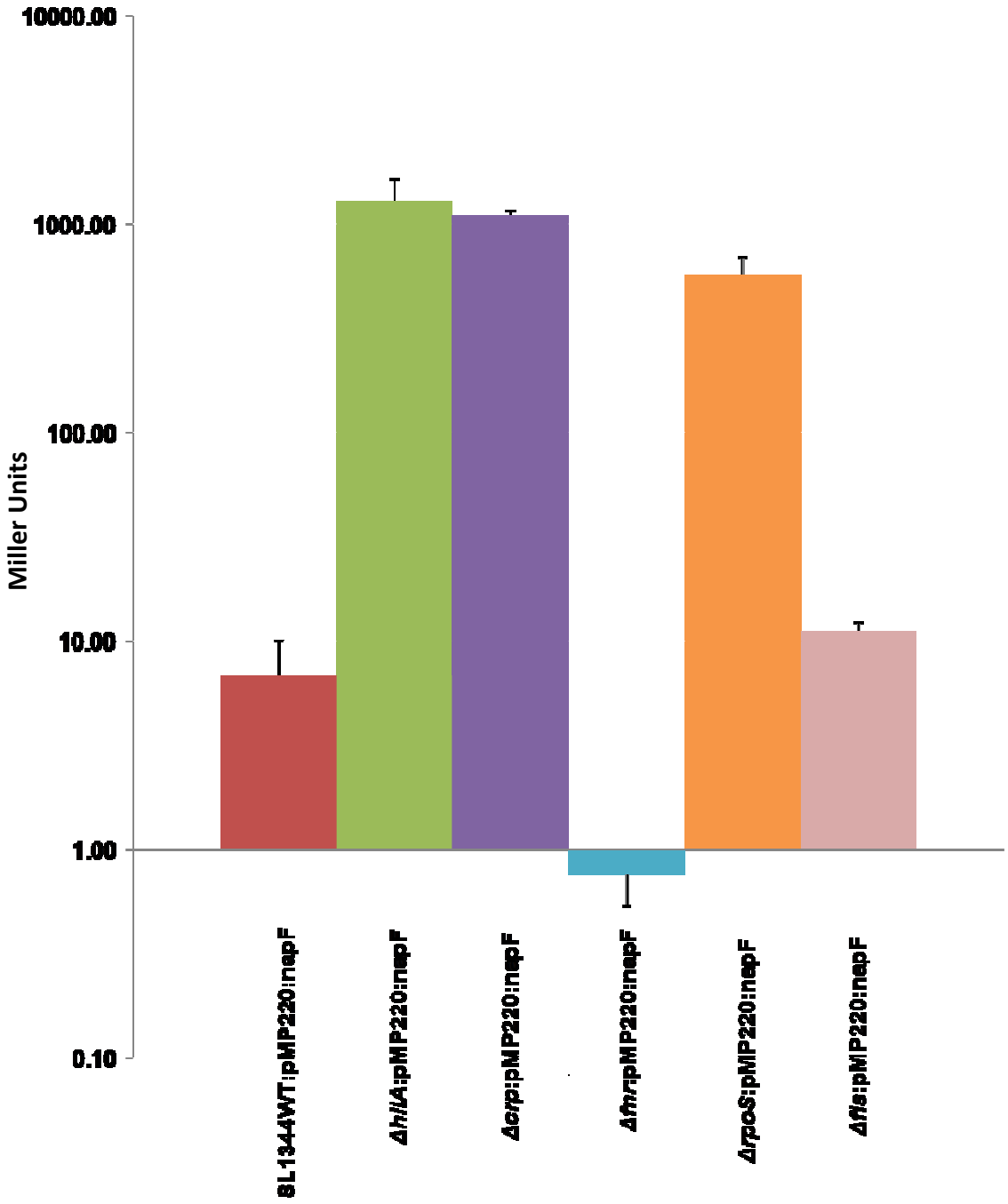


Figure 38 - Anaerobic β-Galactosidase assay pMP220 with napF insert, excluding empty plasmid. Results indicatethat under these conditions, HilA (■), Crp (■), RpoS (■), and to a lesser extent, Fis (■), are negative regulators of NapF. Conversely, under anaerobic conditions, Fnr (■) is a positive regulator of NapF.Miller units for NapF in HilA, Crp, and RpoS background significantly different (student t test, $p<0.05$) to WT. Error bars show the Standard Error of the Mean.

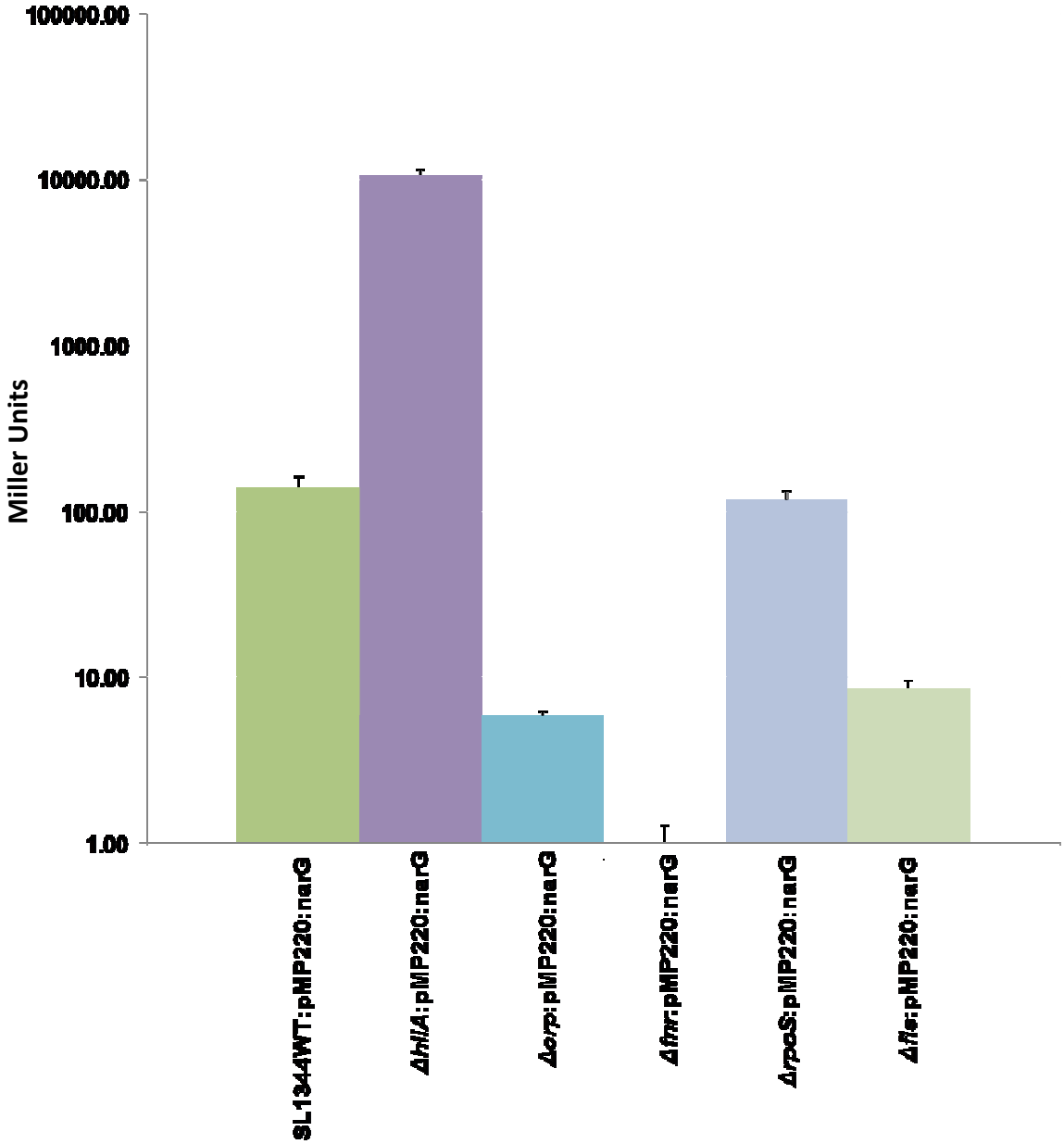


Figure 39 - Anaerobic β-Galactosidase assay, pMP220 plasmid with narG insert, excluding empty plasmid. The results indicate that HilA (■) is the only negative regulator of NarG under these conditions. The effect of RpoS (■) is relatively small, but would be as a positive regulator, while Crp (■), Fnr (■)and Fis (■) all exert a larger positive regulatory role on NarG under anaerobic conditions. Miller units for narG in Fis, Fnr, Crp, and HilA background significantly different (student t test, $p<0.05$) to WT. Error bars show the Standard Error of the Mean.

6.4 Discussion

My results show that the transcription factor FNR (fumarate nitrate reduction) and the alternative sigma factor RpoS both exert positive regulation over NapF, under aerobic conditions. This finding supports the findings of other work which investigated the respective roles of FNR and RpoS in *E.coli* strain MG1655 (Constantinidou, Hobman et al. 2006), though that addressed the transition between aerobic and anaerobic conditions. In addition, there is clearly a change in activity under anaerobic conditions; RpoS switches from positive regulation aerobically, to negative regulation in the absence of oxygen. Other work has also shown that RpoS has different roles in both aerobiosis and anaerobiosis in *E. coli* (King and Ferenci 2005). King and Ferenci also postulate that RpoS expression may not always confer a clear advantage to the bacteria, whilst admitting that RpoS is vital to the virulence of many pathogenic microorganisms, including *S*. Typhimurium (Fang, Libby et al. 1992)

In the case of NarG, it is clear that Fis exerts a very high level of negative control under aerobic conditions, on *S*. Typhimurium. Further, Crp also provides a (much lower) level of negative control over NarG, while HilA, FNR, and RpoS all regulate NarG positively, but at low levels. Crp (cAMP Receptor Protein) is structurally and functionally related to FNR (Spiro, Gaston et al. 1990), and both have been shown to be involved in the switch between aerobic and anaerobic metabolism in *E. coli* (Green, Bennett et al. 1996). In *E.coli*, Fis is recognised as a growth phase dependent regulator (Thompson, Moitoso de Vargas et al. 1987; Schneider, Travers et al. 1997). Genome analysis using *E. coli* has shown that there is little variation in the availability of Fis-binding sites between aerobic and anaerobic conditions, suggesting that in either condition, Fis activity remains fundamentally the same (Cho, Knight et al. 2008). Anaerobically in *S*. Typhimurium, my results indicate that Fis exerts a positive regulatory role on

NarG, the opposite of what was observed under aerobic conditions. HilA is the only negative regulator of NarG in the absence of oxygen; all the other regulators tested would exert positive regulation on NarG in these conditions. NapF, however, undergoes negative regulation by four of the six regulators tested anaerobically, which also includes HilA. Consequently, the findings outlined here lend further weight to the initial observations made by Alston *et al* that the co-expression of genes of the Nap operon with those of SPI-1, and that this occurs under the control of a putative HilA box. HilA is known to activate SPI-1 genes which facilitate invasion of epithelial cells (Bajaj, Hwang et al. 1995). Figure 40 summarises the different regulatory effects in aerobic and anaerobic conditions as measured through the use of β-Galactosidase Assays.

Aerobic / Anaerobic	Insert	Negative Regulation	No effect	Positive Regulation
Aerobic	NapF	*Δcrp* *Δfis*	*ΔhilA*	*Δfnr* *ΔrpoS*
	NarG	*Δcrp* *Δfis*		*ΔhilA* *Δfnr* *ΔrpoS*
Anaerobic	Nap F	*ΔhilA* *Δcrp* *Δfis* *ΔrpoS*		*Δfnr*
	NarG	*ΔhilA*	*ΔrpoS*	*Δcrp* *Δfnr* *Δfis*

Figure 40 - The different effects of regulators in aerobic and anaerobic conditions for pMP220 plasmid with NapF and NarG inserts.

Chapter 7 – General Discussion

As each chapter has already been discussed individually, here I summarise the findings made during my endeavours and make suggestions for future studies.

Salmonella enterica serovar Typhi causes systemic disease in humans. The same route of infection as that employed by *S.* Typhi is also used by *S. enterica* serovar Typhimurium during its infection of mice. This makes the *S.* Typhimurium mouse infection model an ideal one to use in studies investigating the systemic disease in humans.

Nitric oxide is known to be important in the control of infections by a range of pathogens, since mice which are unable to produce NO are more susceptible to infection (Shiloh, MacMicking et al. 1999). The initial stages of this work focused on the importance of NO metabolism in *Salmonella* in relation to virulence. Attention was then turned to how *Salmonella* deals with some of the ordinarily potentially toxic products of NO metabolism, like other reactive nitrogen species such as nitrate and the nitrate reductase machinery it employs to neutralise their effects.

7.1 NorV, NrfA and HmpA

Under the conditions which were tested, the SL1344 *ΔnorVΔnrfAΔhmpA* triple deletion mutant was no more or less affected than the isogenic parent. Rowley *et al* (unpublished data) had also found no difference between this mutant and the wild type strain during trials using intraperitoneal infection techniques in Balb/C mice. Our findings support the hypothesis that other mechanisms for NO detoxification exist which are induced during animal infection, and which remain to be elucidated.

S. Typhimurium proliferation increases during an infection if NO production by the innate immune system of mice is restricted (Umezawa, Akaike et al. 1997; MacFarlane, Schwacha et al. 1999). The ability of mutant strains of *Salmonella* to

downregulate host NO production therefore seems to be favourable and has also been demonstrated (Eriksson, Bjorkman et al. 2000). Several pathogens, as part of their normal infection strategy, survive intracellularly within their host, so it is to their advantage to circumvent attempts by the host immune system to eradicate them whilst there. Both *S*. Typhimurium and *Mycobacterium tuberculosis*, while inducing iNOS production in macrophages, can detoxify both ROS and RNI. *S*. Typhimurium interferes with the recruitment of components required by phagocytic host cells to mount their defensive burst (Vazquez-Torres, Xu et al. 2000).

Das *et al* have also shown recently that transporters of nitrogen species are crucial for controlling what happens between NO and host defences during the resistance of the host to *Salmonella* infection (Das, Lahiri et al. 2009). Production of NO in response to IFN-γ activation of RAW264.7 macrophages is inhibited in macrophages infected with wild-type *Salmonella*, and the nitrite transporter nirC was found to be fundamental to this effect (Das, Lahiri et al. 2009). This shows that some bacterial species, including *Salmonella*, can defend themselves from NO stress by inhibiting the ability of the mammalian cells containing them to produce NO. Consequently, the pathogens prevent the function of one of the main resources available to the mammalian immune response.

Future work which is intimated should include complementation of the strains which were developed, together with complete sequencing of them. Also, the possible impact of alterations on the polarity of the genes involved should be more fully explored by testing for unmarked deletions.

7.2 Nitrate reductases, Nap and Nar

In both nutrient replete medium (LB) and minimal medium containing glucose (MGM), the growth of SL1344 nitrate reductase mutants is similar to that of

SL1344 wild type, regardless of whether the conditions for growth were aerobic or anaerobic.

The Cole group has shown that using media with glycerol rather than glucose as the carbon source and supplemented with nitrate and fumarate induced expression of *nrfA* in *E.coli*(Darwin, Hussain et al. 1993). However, supplementation of this respiratory electron acceptor is not required for nitrate reductases, since they do not depend on fumarate. We adapted the Glycerol Nitrate Fumarate (GNF) medium developed by Darwin *et al* by removing the fumarate, to assess the ability of the nitrate reductase mutants to grow in the presence of replete nitrate. The nitrate reductase mutants were tested in anaerobic conditions using this medium. It was interesting that the SL1344 *ΔnarGHJI* (NR-A nitrate reductase system) single deletion mutant consistently and repeatedly showed a significant growth reduction compared to the wild type and other two single mutants in this medium. Other work has found that a *narG* mutant of *M. tuberculosis* also fails to grow in nitrate replete broth media (Malm, Tiffert et al. 2009). A comparison was also made between SL1344 Wild Type and the *ΔnarGHJI* single and *ΔnarGHJIΔnarZYWVΔnapDA* triple nitrate reductase deletion mutants. Growth inhibition of the single mutant was demonstrated and also confirmed that growth of the triple deletion mutant was even more reduced. As growth of the triple deletion mutant was even more inhibited than the single *ΔnarGHJI* mutant, we concluded that more than the NR-A nitrate reductase system is involved in nitrate reduction in this medium. From further work, it was possible to conclude that loss of both the NR-A and Nap nitrate reductase systems together prevents growth almost completely, accounting for the entire adverse effect on growth observed in the triple nitrate reductase system deletion mutant. Sohaskey and Malm have separately drawn similar conclusions from their work on *M. tuberculosis* and *E.coli* respectively, both showing that NarGHJI mutants were disadvantaged in replete nitrate under anaerobic conditions

(Sohaskey 2008; Malm, Tiffert et al. 2009). The NR-A system is therefore capable of compensating for the loss of both the NR-Z and Nap systems. This is not a reciprocal arrangement, as the Nap system does not compensate completely for the loss of the NR-A system. This suggests that the complete loss of growth in the triple mutant is in fact due to the loss of the NR-A and Nap systems, and not loss of the NR-Z system. The addition of fumarate back into the medium made no difference to the previous results and did not rescue growth in NR-A and Nap mutants.

We used MM5.8 medium which is considered a good model for the intracellular environment of the SCV inside mammalian macrophages within which *Salmonella* not only survives but also replicates. Many intracellular pathogens are specially adapted to withstand the rigours of the harsh environment found within macrophages (Lavigne, O'Callaghan et al. 2005), and particularly within the phagosome itself. Indeed, several, such as *Toxoplasma, Leishmania, Mycobacterium* and *Legionella* and including *Salmonella spp*., are exquisitely adapted to actually thrive in such surroundings (Amer and Swanson 2002). In *Salmonella*, we found no difference between the growth patterns observed for the SL1344 single, double or triple nitrate reductase mutant when compared to the SL1344 wild type strain in MM5.8 medium. This suggests that *Salmonella* has such a wide range of strategies to deal with the rigours of the macrophage attack that deletion of known nitrate reductase systems does not compromise bacterial survival.

By carrying out motility assays on the nitrate reductase mutants, we have confirmed that loss of the nitrate reductase systems in *S*. Typhimurium does not compromise motility. Previously, other work had shown that both *Pseudomonas aeruginosa and E. coli* suffer from motility and swarming defects when the activity of their Nar nitrate reductase or Hmp respectively is compromised (Schreiber, Krieger et al. 2007; Stevanin, Read et al. 2007) This was attributed to the loss of

flagellae probably due to the onerous metabolic burden placed on the bacteria by synthesis of Hmp in the absence of NO rather than due to Nar.

S. Typhimurium uses SPI-1 to invade epithelial cells, (Shea *et al*., 1996) and Alston *et al.* have found that the SPI-1 genes are co-expressed with other genes of the *nap* operon (unpublished data) as outlined earlier. We postulated that the *nap* operon is important in the early stages of a *Salmonella* infection. Since the *nap* operon encodes one of the three nitrate reductase systems of *Salmonella*, Nap, we investigated the possibility of a direct link between invasion and nitrate reduction. Invasion assays of epithelial cells provided a good model to explore this connection.

We found that invasion of the double mutants varied little from the wild type and the triple mutant was more invasive. However, once inside the epithelial cells, there was no difference between the replicative ability of any of the nitrate reductase mutants compared to the SL1344 wild type strain.

Entry of *Salmonella* into epithelial type cells of the gastrointestinal tract requires the deployment of the SPI-1 TTSS apparatus. In macrophages, there is no need for *Salmonella* to use SPI-1 to enter the cells, since the phagocytic nature of the macrophage means that it will actively seek to engulf the bacteria. We found that the triple mutant was more invasive in human epithelial cells than wild type, whether or not SPI-1 TTSS had been induced prior to infection.

This interesting finding was followed up by carrying out work *in vivo* to see whether the increased virulence would also be manifest in a mouse model of infection. Oral infection of Balb/C mice was used as the preferred method, since this would be closer to the natural *in vivo* method of infection by *Salmonella*. In the initial stages of the infection, during the passage and occupation of the murine stomach, intestine and colon by the bacteria, there was little difference between the numbers of the mutant strain bacteria which were isolated and the wild type strain, However, following advancement of the infection to the systemic

phase, the mutant demonstrated increased virulence over the wild type. It has recently been confirmed that both the membrane bound nitrate reductase (Nar) and the periplasmic nitrate reductase (Nap) are instrumental in the endogenous production of both NO and nitrous oxide (N_2O) (Rowley, Hensen et al. 2012).

The removal of all three nitrate reductase systems may have consequently reduced the levels of NO and N_2O which are normally produced endogenously by these systems. This would in turn have reduced the environmental challenge to these bacteria, so that more were able to expend energy in survival and replicative functions, increasing their numbers present in the systemic samples.

Interpretation of our results lead to the conclusion that nitrate reductase mutants, of the Nar and Nap operon systems, are not compromised in their ability to employ the SPI-1 TTSS to invade epithelial cells of the mammalian gastrointestinal tract.

SPI-1 mutants are still able to infect the host following intraperitoneal (IP), infection to the same extent as wild type *Salmonella* (Galan 1996; Ochman, Soncini *et al*. 1996). Further, Spi-1 is not necessary for intracellular survival and the systemic phase of any infection. However, SPI-1 mutants fail to infect the host following oral infection, and also are not able to invade enterocytes *in vitro* (Lucas and Lee 2001). This supports the premise that SPI-1 is required for invasion via the gut epithelium. In this study, it was found that nitrate reductase mutants were still able to invade via the gut epithelium; it is therefore possible to extrapolate that loss of the nitrate reductase systems does not also compromise expression of the components required for production and assembly of the SPI-1 TTSS. Consequently, despite the findings of Alston *et al* that genes of the *nap* operon are co-expressed with those of the SPI-1 TTSS, the findings from this work suggest that it is not because Nap genes are required for the efficient deployment of SPI-1.

It seems that the nitrate reductase systems do, however, ameliorate the replicative capacity of *Salmonella* within macrophages. Loss of the nitrate reductase systems causes an increase in bacterial replication. The mechanisms with which the nitrate reductases might suppress replication are worthy of further investigation, since it might be possible to further exploit them in the design of effective treatments for the illnesses caused by *Salmonella* infections whilst limiting replication. The reduction in the endogenous NO production may provide an interesting place to start. The nitrate reductase systems appear to place a brake on the ability of *Salmonella* to overwhelm the host defences. This may help to ensure that the infection lasts long enough to ensure that it is passed on before the host is destroyed. Several pathogens are known to control their potential for destruction of their host to ensure their own survival. Consideration of the evolutionary advantage which is or may be available to organisms which control their virulence supports the view that there is a close relationship between virulence and the effect on host mortality (Lenski and May 1994; Koch 2007)

In summary, the results of this work suggest that what has been identified is a highly replicative phenotype in the nitrate reductase mutants of *Salmonella* Typhimurium rather than a phenotype which is capable of highly increased invasion levels when ingested via the oral route.

Findings from the β-Galactosidase Assays carried out support the findings of other work which investigated the respective roles of FNR and RpoS in *E.coli* strain MG1655 (Constantinidou, Hobman et al. 2006). This work confirmed that RpoS switches from positive regulation aerobically, to negative regulation in the absence of oxygen. Other work has also shown that RpoS has different roles in both aerobiosis and anaerobiosis (King and Ferenci 2005). King and Ferenci postulate that RpoS expression may not always confer a clear advantage to the bacteria, although RpoS is vital to the virulence of many pathogenic microorganisms, including *S*. Typhimurium (Fang, Libby et al. 1992).

In the case of NarG, it was shown that Fis exerts a high level of negative control under aerobic conditions, on *S*. Typhimurium. Crp also provides a (much lower) level of negative control over NarG, while HilA, FNR, and RpoS all regulate NarG positively, albeit at low levels. Crp (cAMP Receptor Protein) is structurally and functionally related to FNR (Spiro, Gaston et al. 1990), and both are involved in the switch between aerobic and anaerobic metabolism in *E. coli* (Green, Bennett et al. 1996). In *E.coli*, Fis is recognised as a growth phase dependent regulator (Thompson, Moitoso de Vargas et al. 1987; Schneider, Travers et al. 1997). Genome analysis using *E. coli* has shown that there is little variation in the availability of Fis-binding sites between aerobic and anaerobic conditions, suggesting that in either condition, Fis activity remains fundamentally the same (Cho, Knight et al. 2008). However, this work indicates that anaerobically in *S*. Typhimurium, Fis exerts a positive regulatory role on NarG, the opposite of what was observed under aerobic conditions. HilA is the only negative regulator of NarG in the absence of oxygen; all the other regulators tested exert positive regulation on NarG in these conditions. NapF, however, undergoes negative regulation by four of the six regulators tested anaerobically, which also includes HilA. Consequently, the findings outlined here lend further weight to the initial observations made by Alston *et al* of the co-expression of genes of the Nap operon with those of SPI-1, and that this occurs under the control of a putative HilA box. HilA is known to activate SPI-1 genes which facilitate invasion of epithelial cells (Bajaj, Hwang et al. 1995). However, this co-expression it seems, is incidental, and is not reflected by any effect on invasion in nitrate reductase, and particularly Nap, mutants.

Other future work to pinpoint whether HilA does indeed regulate expression of Nap, employing chIP-on-chip and Electrophoretic Mobility Shift Assay (EMSA) techniques, would be beneficial. If Nap were found to be regulated in this way, further work would also be indicated to explore why that is advantageous to the

organism, and enzyme assays could be useful in this respect. Reference to work by the Bäumler group suggests that in a colitis mouse model, the interaction may be linked to SPI-1 expression (Winter, Thiennimitr et al. 2010). It would be interesting to carry out green fluorescent protein (gfp) fusions to *nap* and *nar*, followed by confocal microscopy techniques to explore further the conditions under which expression of the nitrate and nitrite reductases are switched on or off. It would also be useful to conduct further invasion and protection assays using colonic cell lines, such as Caco-2 or HT29 human cells. However, since these are already compromised by virtue of their tumourigenic and immortal nature, even better would be cells of the normal mucosa, such as NCM460, although these present more difficulties due to their fussy culture requirements.

Overall, however, the conclusion reached as a result of the work carried out in this study is that the *nap* operon which encodes the periplasmic nitrate reductase in *Salmonella* does not play a role in the early process of *Salmonella* infection.

References

Abdool Gaffar, M. S., Y. K. Seedat, et al. (1992). "The white cell count in typhoid fever." Trop Geogr Med **44**(1-2): 23-7.
Abergel, C., V. Monchois, et al. (2007). "Structure and evolution of the Ivy protein family, unexpected lysozyme inhibitors in Gram-negative bacteria." Proc Natl Acad Sci U S A **104**(15): 6394-9.
Acharya, I. L., C. U. Lowe, et al. (1987). "Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of Salmonella typhi. A preliminary report." N Engl J Med **317**(18): 1101-4.
Adak, G. K., S. M. Meakins, et al. (2005). "Disease risks from foods, England and Wales, 1996-2000." Emerg Infect Dis **11**(3): 365-72.
Aliabadi, Z., F. Warren, et al. (1986). "Oxygen-regulated stimulons of Salmonella typhimurium identified by Mu d(Ap lac) operon fusions." J Bacteriol **165**(3): 780-6.
Allal, R., B. Kastler, et al. (1993). "Splenic abscesses in typhoid fever: US and CT studies." J Comput Assist Tomogr **17**(1): 90-3.
Amer, A. O. and M. S. Swanson (2002). "A phagosome of one's own: a microbial guide to life in the macrophage." Curr Opin Microbiol **5**(1): 56-61.
Anderson, E. S. (1968). "Drug resistance in Salmonella typhimurium and its implications." Br Med J **3**(5614): 333-9.
Andersson, D. I. and B. R. Levin (1999). "The biological cost of antibiotic resistance." Curr Opin Microbiol **2**(5): 489-93.
Ansaldi, M., L. Theraulaz, et al. (2007). "Aerobic TMAO respiration in Escherichia coli." Mol Microbiol **66**(2): 484-94.
Arques, J. L., I. Hautefort, et al. (2009). "Salmonella induces flagellin- and MyD88-dependent migration of bacteria-capturing dendritic cells into the gut lumen." Gastroenterology **137**(2): 579-87, 587 e1-2.
Azad, A. K., R. Islam, et al. (1997). "Comparison of clinical features and pathologic findings in fatal cases of typhoid fever during the initial and later stages of the disease." Am J Trop Med Hyg **56**(5): 490-3.
Backhed, F., R. E. Ley, et al. (2005). "Host-bacterial mutualism in the human intestine." Science **307**(5717): 1915-20.
Baggesen, D. L. and F. M. Aarestrup (1998). "Characterisation of recently emerged multiple antibiotic-resistant Salmonella enterica serovar typhimurium DT104 and other multiresistant phage types from Danish pig herds." Vet Rec **143**(4): 95-7.
Bajaj, V., C. Hwang, et al. (1995). "hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes." Mol Microbiol **18**(4): 715-27.
Bajaj, V., R. L. Lucas, et al. (1996). "Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression." Mol Microbiol **22**(4): 703-14.
Baker-Austin, C., M. S. Wright, et al. (2006). "Co-selection of antibiotic and metal resistance." Trends Microbiol **14**(4): 176-82.
Bamford, V. A., H. C. Angove, et al. (2002). "Structure and spectroscopy of the periplasmic cytochrome c nitrite reductase from Escherichia coli." Biochemistry **41**(9): 2921-31.
Bang, I. S., L. Liu, et al. (2006). "Maintenance of nitric oxide and redox homeostasis by the salmonella flavohemoglobin hmp." J Biol Chem **281**(38): 28039-47.
Baquero, F. a. B., J. (1997). "Evolution of Antibiotic Resistance." Trends in Ecology and Evolution **12**(12): 482-487.
Barrett, E. L. and D. L. Riggs (1982). "Evidence of a second nitrate reductase activity that is distinct from the respiratory enzyme in Salmonella typhimurium." J Bacteriol **150**(2): 563-71.
Basu, D. and M. Herlyn (2008). "Salmonella typhimurium as a novel RNA interference vector for cancer gene therapy." Cancer Biol Ther **7**(1): 151-2.
Beaber, J. W., B. Hochhut, et al. (2004). "SOS response promotes horizontal dissemination of antibiotic resistance genes." Nature **427**(6969): 72-4.

Becker, D., M. Selbach, et al. (2006). "Robust Salmonella metabolism limits possibilities for new antimicrobials." Nature **440**(7082): 303-7.
Benjamin, N., F. O'Driscoll, et al. (1994). "Stomach NO synthesis." Nature **368**(6471): 502.
Berg, D. T., A. Gupta, et al. (2007). "Negative Regulation of Inducible Nitric-oxide Synthase Expression Mediated through Transforming Growth Factor- -dependent Modulation of Transcription Factor TCF11." J Biol Chem **282**(51): 36837-44.
Berks, B. C., D. J. Richardson, et al. (1995). "The napEDABC gene cluster encoding the periplasmic nitrate reductase system of Thiosphaera pantotropha." Biochem J **309 (Pt 3)**: 983-92.
Berks, B. C., D. J. Richardson, et al. (1994). "Purification and characterization of the periplasmic nitrate reductase from Thiosphaera pantotropha." Eur J Biochem **220**(1): 117-24.
Bertero, M. G., R. A. Rothery, et al. (2005). "Structural and biochemical characterization of a quinol binding site of Escherichia coli nitrate reductase A." J Biol Chem **280**(15): 14836-43.
Bertero, M. G., R. A. Rothery, et al. (2003). "Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A." Nat Struct Biol **10**(9): 681-7.
Beuzon, C. R., G. Banks, et al. (1999). "pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella typhimurium." Mol Microbiol **33**(4): 806-16.
Beuzon, C. R. and D. W. Holden (2001). "Use of mixed infections with Salmonella strains to study virulence genes and their interactions in vivo." Microbes Infect **3**(14-15): 1345-52.
Bhan, M. K., R. Bahl, et al. (2005). "Typhoid and paratyphoid fever." Lancet **366**(9487): 749-62.
Bjorne, H. H., J. Petersson, et al. (2004). "Nitrite in saliva increases gastric mucosal blood flow and mucus thickness." J Clin Invest **113**(1): 106-14.
Blasco, F., J. P. Dos Santos, et al. (1998). "NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of Escherichia coli." Mol Microbiol **28**(3): 435-47.
Blasco, F., C. Iobbi, et al. (1990). "Nitrate reductases of Escherichia coli: sequence of the second nitrate reductase and comparison with that encoded by the narGHJI operon." Mol Gen Genet **222**(1): 104-11.
Blasco, F., J. Pommier, et al. (1992). "Involvement of the narJ or narW gene product in the formation of active nitrate reductase in Escherichia coli." Mol Microbiol **6**(2): 221-30.
Blazquez, J., A. Oliver, et al. (2002). "Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance?" Curr Drug Targets **3**(4): 345-9.
Bochner, B. R. (1984). Curing bacterial cells of lysogenic viruses by using UCB indicator plates, Biotechniques. Vol. 2, no. 4, pp. 234-240. 1984.
Bockman, D. E. and M. D. Cooper (1973). "Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study." Am J Anat **136**(4): 455-77.
Bodenmiller, D. M. and S. Spiro (2006). "The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator." J Bacteriol **188**(3): 874-81.
Bohnert, J., B. Hubner, et al. (2000). "Rapid identification of Enterobacteriaceae using a novel 23S rRNA-targeted oligonucleotide probe." Int J Hyg Environ Health **203**(1): 77-82.
Bonnefoy, V. and J. A. Demoss (1994). "Nitrate reductases in Escherichia coli." Antonie Van Leeuwenhoek **66**(1-3): 47-56.
Borisov, V. B., E. Forte, et al. (2004). "Interaction of the bacterial terminal oxidase cytochrome bd with nitric oxide." FEBS Lett **576**(1-2): 201-4.
Borrelli, L., A. Fioretti, et al. (2010). "Salmonella typhimurium DT104 in farmed rabbits." J Vet Med Sci **73**(3): 385-7.

Borries, G. V. T. (1930). "On nose-bleeding." The Journal of Laryngology & Otology, 45 , pp 81-94

Bourret, T. J., S. Porwollik, et al. (2008). "Nitric oxide antagonizes the acid tolerance response that protects Salmonella against innate gastric defenses." PLoS ONE **3**(3): e1833.

Bowden, S. D., V. K. Ramachandran, et al. (2010). "An incomplete TCA cycle increases survival of Salmonella Typhimurium during infection of resting and activated murine macrophages." PLoS One **5**(11): e13871.

Bowden, S. D., G. Rowley, et al. (2009). "Glucose and glycolysis are required for the successful infection of macrophages and mice by Salmonella enterica serovar typhimurium." Infect Immun **77**(7): 3117-26.

Boyd, E. F., F. S. Wang, et al. (1996). "Molecular genetic relationships of the salmonellae." Appl Environ Microbiol **62**(3): 804-8.

Braddock, M., A. Quinn, et al. (2004). "Therapeutic potential of targeting IL-1 and IL-18 in inflammation." Expert Opin Biol Ther **4**(6): 847-60.

Brennan, M. A. and B. T. Cookson (2000). "Salmonella induces macrophage death by caspase-1-dependent necrosis." Mol Microbiol **38**(1): 31-40.

Breton, J., B. C. Berks, et al. (1994). "Characterization of the paramagnetic iron-containing redox centres of Thiosphaera pantotropha periplasmic nitrate reductase." FEBS Lett **345**(1): 76-80.

Brige, A., D. Leys, et al. (2002). "The 1.25 A resolution structure of the diheme NapB subunit of soluble nitrate reductase reveals a novel cytochrome c fold with a stacked heme arrangement." Biochemistry **41**(15): 4827-36.

Briggs, C. E. and P. M. Fratamico (1999). "Molecular characterization of an antibiotic resistance gene cluster of Salmonella typhimurium DT104." Antimicrob Agents Chemother **43**(4): 846-9.

Browning, D. F., D. J. Lee, et al. (2010). "Down-regulation of the Escherichia coli K-12 nrf promoter by binding of the NsrR nitric oxide-sensing transcription repressor to an upstream site." J Bacteriol **192**(14): 3824-8.

Bryan, N. S. (2006). "Nitrite in nitric oxide biology: cause or consequence? A systems-based review." Free Radic Biol Med **41**(5): 691-701.

Buchmeier, N., A. Blanc-Potard, et al. (2000). "A parallel intraphagosomal survival strategy shared by mycobacterium tuberculosis and Salmonella enterica." Mol Microbiol **35**(6): 1375-82.

Bulling, E., R. Stephan, et al. (1973). "[The development of antibiotics resistance among salmonella bacteria of animal origin in the Federal Republic of Germany and West Berlin. 1st communication: a comparison between the years of 1961 and 1970-71 (author's transl)]." Zentralbl Bakteriol Orig A **225**(2): 245-56.

Burlat, B., J. D. Gwyer, et al. (2005). "Cytochrome c nitrite reductase: from structural to physicochemical analysis." Biochem Soc Trans **33**(Pt 1): 137-40.

Busse, R. and A. Mulsch (1990). "Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells." FEBS Lett **275**(1-2): 87-90.

C.D.C (2008) "Investigation of Outbreak of Infections Caused by Salmonella Saintpaul " http://www.cdc.gov/salmonella/saintpaul/

C.D.C. (2009) "Investigation of an Outbreak of Salmonella Saintpaul Infections Linked to Raw Alfalfa Sprouts." http://www.cdc.gov/salmonella/saintpaul/alfalfa/

Campbell, W. H. and K. R. Kinghorn (1990). "Functional domains of assimilatory nitrate reductases and nitrite reductases." Trends Biochem Sci **15**(8): 315-9.

Cano, D. A., M. Martinez-Moya, et al. (2001). "Salmonella enterica serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation." Infect Immun **69**(10): 6463-74.

Carter, P. B. and F. M. Collins (1974). "The route of enteric infection in normal mice." J Exp Med **139**(5): 1189-203.

Cavallaro, E., K. Date, et al. (2011). "Salmonella typhimurium infections associated with peanut products." N Engl J Med **365**(7): 601-10.

Chakravortty, D. (2010). "*Salmonella* vaccine: a black sheep " Current Science **98**(2): 149-150.
Chakravortty, D., I. Hansen-Wester, et al. (2002). "Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates." J Exp Med **195**(9): 1155-66.
Chakravortty, D. and M. Hensel (2003). "Inducible nitric oxide synthase and control of intracellular bacterial pathogens." Microbes Infect **5**(7): 621-7.
Chan, K., S. Baker, et al. (2003). "Genomic comparison of Salmonella enterica serovars and Salmonella bongori by use of an S. enterica serovar typhimurium DNA microarray." J Bacteriol **185**(2): 553-63.
Chang, L., L. I. Wei, et al. (1999). "Expression of the Escherichia coli NRZ nitrate reductase is highly growth phase dependent and is controlled by RpoS, the alternative vegetative sigma factor." Mol Microbiol **34**(4): 756-66.
Chen, L. M., S. Hobbie, et al. (1996). "Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses." Science **274**(5295): 2115-8.
Chen, L. M., K. Kaniga, et al. (1996). "Salmonella spp. are cytotoxic for cultured macrophages." Mol Microbiol **21**(5): 1101-15.
Cherepanov, P. P. and W. Wackernagel (1995). "Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant." Gene **158**(1): 9-14.
Chin'ombe, N., W. R. Bourn, et al. (2009). "Oral vaccination with a recombinant Salmonella vaccine vector provokes systemic HIV-1 subtype C Gag-specific CD4+ Th1 and Th2 cell immune responses in mice." Virol J **6**: 87.
Cho, B. K., E. M. Knight, et al. (2008). "Genome-wide analysis of Fis binding in Escherichia coli indicates a causative role for A-/AT-tracts." Genome Res **18**(6): 900-10.
CIDRAP (2007). Drug-resistant Salmonella outbreak linked to ground beef, Center for Infectious Disease Research and Policy, University of Minnesota.
CIDRAP (2010). USDA estimates E coli, Salmonella costs at $3.1 billion, Center for Infectious Disease Research and Policy, University of Minnesota.
Clarke, T. A., G. L. Kemp, et al. (2008). "Role of a Conserved Glutamine Residue in Tuning the Catalytic Activity of Escherichia coli Cytochrome c Nitrite Reductase." Biochemistry **47**(12): 3789-3799.
Clegg, S. J., W. Jia, et al. (2006). "Role of the Escherichia coli nitrate transport protein, NarU, in survival during severe nutrient starvation and slow growth." Microbiology **152**(Pt 7): 2091-100.
Coburn, B., G. A. Grassl, et al. (2007). "Salmonella, the host and disease: a brief review." Immunol Cell Biol **85**(2): 112-8.
Cohen, H. J., S. M. Mechanda, et al. (1996). "PCR amplification of the fimA gene sequence of Salmonella typhimurium, a specific method for detection of Salmonella spp." Appl Environ Microbiol **62**(12): 4303-8.
Constantinidou, C., J. L. Hobman, et al. (2006). "A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as Escherichia coli K12 adapts from aerobic to anaerobic growth." J Biol Chem **281**(8): 4802-15.
Contreras, I., C. S. Toro, et al. (1997). "Salmonella typhi mutants defective in anaerobic respiration are impaired in their ability to replicate within epithelial cells." Microbiology **143 (Pt 8)**: 2665-72.
Cookson, B. T. and M. A. Brennan (2001). "Pro-inflammatory programmed cell death." Trends Microbiol **9**(3): 113-4.
Cornes, J. S. (1965). "Number, size, and distribution of Peyer's patches in the human small intestine: Part I The development of Peyer's patches." Gut **6**(3): 225-9.
Crawford, M. J. and D. E. Goldberg (1998). "Regulation of the Salmonella typhimurium flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide." J Biol Chem **273**(51): 34028-32.

Crosa, J. H., D. J. Brenner, et al. (1973). "Molecular relationships among the Salmonelleae." J Bacteriol **115**(1): 307-15.
Crump, J. A., S. P. Luby, et al. (2004). "The global burden of typhoid fever." Bull World Health Organ **82**(5): 346-53.
Cruz-Garcia, C., A. E. Murray, et al. (2007). "Respiratory nitrate ammonification by Shewanella oneidensis MR-1." J Bacteriol **189**(2): 656-62.
Cryz, S. J. and E. Furer (1988). "Further characterization of the Salmonella typhi Ty21a vaccine strain." J Infect Dis **157**(6): 1276-9.
Curtiss, R., 3rd, J. E. Galan, et al. (1990). "Stabilization of recombinant avirulent vaccine strains in vivo." Res Microbiol **141**(7-8): 797-805.
Cvjetanovic, B. and K. Uemura (1965). "The Present Status of Field and Laboratory Studies of Typhoid and Paratyphoid Vaccines with Special Reference to Studies Sponsored by World Health Organization." Bull World Health Organ **32**: 29-36.
D.E.F.R.A. (2005). Zoonoses Report. F. a. R. A. Department for Environment. London, D.E.F.R.A.
Darwin, A., H. Hussain, et al. (1993). "Regulation and sequence of the structural gene for cytochrome c552 from Escherichia coli: not a hexahaem but a 50 kDa tetrahaem nitrite reductase." Mol Microbiol **9**(6): 1255-65.
Darwin, K. H. and V. L. Miller (1999). "Molecular basis of the interaction of Salmonella with the intestinal mucosa." Clin Microbiol Rev **12**(3): 405-28.
Das, P., A. Lahiri, et al. (2009). "Novel role of the nitrite transporter NirC in Salmonella pathogenesis: SPI2-dependent suppression of inducible nitric oxide synthase in activated macrophages." Microbiology **155**(Pt 8): 2476-89.
Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products." Proc Natl Acad Sci U S A **97**(12): 6640-5.
Davis, M. A., D. D. Hancock, et al. (1999). "Changes in antimicrobial resistance among Salmonella enterica Serovar typhimurium isolates from humans and cattle in the Northwestern United States, 1982-1997." Emerg Infect Dis **5**(6): 802-6.
Deplancke, B. and H. R. Gaskins (2001). "Microbial modulation of innate defense: goblet cells and the intestinal mucus layer." Am J Clin Nutr **73**(6): 1131S-1141S.
DeRoeck, D., L. Jodar, et al. (2007). "Putting typhoid vaccination on the global health agenda." N Engl J Med **357**(11): 1069-71.
Detweiler, C. S., D. B. Cunanan, et al. (2001). "Host microarray analysis reveals a role for the Salmonella response regulator phoP in human macrophage cell death." Proc Natl Acad Sci U S A **98**(10): 5850-5.
Devico, A. L., T. R. Fouts, et al. (2002). "Development of an oral prime-boost strategy to elicit broadly neutralizing antibodies against HIV-1." Vaccine **20**(15): 1968-74.
Diao, J., Y. Zhang, et al. (2008). "Crystal structure of SopA, a Salmonella effector protein mimicking a eukaryotic ubiquitin ligase." Nat Struct Mol Biol **15**(1): 65-70.
DiGiandomenico, A., J. Rao, et al. (2007). "Intranasal immunization with heterologously expressed polysaccharide protects against multiple Pseudomonas aeruginosa infections." Proc Natl Acad Sci U S A **104**(11): 4624-9.
Dougan, G., S. Chatfield, et al. (1988). "Construction and characterization of vaccine strains of Salmonella harboring mutations in two different aro genes." J Infect Dis **158**(6): 1329-35.
du Plessis, J. P., K. Govendrageloo, et al. (1997). "Right-sided endocarditis due to Salmonella typhi." Pediatr Cardiol **18**(6): 443-4.
Dykhuizen, R. S., R. Frazer, et al. (1996). "Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense." Antimicrob Agents Chemother **40**(6): 1422-5.
E.F.S.A. (2008). "Report of the Task Force on Zoonoses Data Collection on the Analysis of the baseline survey on the prevalence of Salmonella in slaughter pigs, in the EU, 2006-2007 [1] - Part A: Salmonella prevalence estimates."

Edwards, P. R. and F. Kauffmann (1952). "A simplification of the Kauffmann-White schema." Am J Clin Pathol **22**(7): 692-7.
Edwards, R. A., G. J. Olsen, et al. (2002). "Comparative genomics of closely related salmonellae." Trends Microbiol **10**(2): 94-9.
Elewaut, D., J. A. DiDonato, et al. (1999). "NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria." J Immunol **163**(3): 1457-66.
Ellermeier, C. D., A. Janakiraman, et al. (2002). "Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria." Gene **290**(1-2): 153-61.
Elvers, K. T., S. M. Turner, et al. (2005). "NssR, a member of the Crp-Fnr superfamily from Campylobacter jejuni, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin." Mol Microbiol **57**(3): 735-50.
Enright, M. C., D. A. Robinson, et al. (2002). "The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA)." Proc Natl Acad Sci U S A **99**(11): 7687-92.
Eriksson, S., J. Bjorkman, et al. (2000). "Salmonella typhimurium mutants that downregulate phagocyte nitric oxide production." Cell Microbiol **2**(3): 239-50.
Eriksson, S., S. Lucchini, et al. (2003). "Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica." Mol Microbiol **47**(1): 103-18.
Ernst, R. K., D. M. Dombroski, et al. (1990). "Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by Salmonella typhimurium." Infect Immun **58**(6): 2014-6.
Everest, P., P. Griffiths, et al. (1995). "Live Salmonella vaccines as a route towards oral immunisation." Biologicals **23**(2): 119-24.
Everest, P., J. Wain, et al. (2001). "The molecular mechanisms of severe typhoid fever." Trends Microbiol **9**(7): 316-20.
Falconi, M., G. Prosseda, et al. (2001). "Involvement of FIS in the H-NS-mediated regulation of virF gene of Shigella and enteroinvasive Escherichia coli." Mol Microbiol **42**(2): 439-52.
Fang, F. C. (1997). "Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity." J Clin Invest **99**(12): 2818-25.
Fang, F. C., S. J. Libby, et al. (1992). "The alternative sigma factor katF (rpoS) regulates Salmonella virulence." Proc Natl Acad Sci U S A **89**(24): 11978-82.
Faucher, S. P., S. Porwollik, et al. (2006). "Transcriptome of Salmonella enterica serovar Typhi within macrophages revealed through the selective capture of transcribed sequences." Proc Natl Acad Sci U S A **103**(6): 1906-11.
Fedtke, I., A. Kamps, et al. (2002). "The nitrate reductase and nitrite reductase operons and the narT gene of Staphylococcus carnosus are positively controlled by the novel two-component system NreBC." J Bacteriol **184**(23): 6624-34.
Filenko, N., S. Spiro, et al. (2007). "The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase." J Bacteriol **189**(12): 4410-7.
Fink, R. C., M. R. Evans, et al. (2007). "FNR is a global regulator of virulence and anaerobic metabolism in Salmonella enterica serovar Typhimurium (ATCC 14028s)." J Bacteriol **189**(6): 2262-73.
Fink, S. L. and B. T. Cookson (2007). "Pyroptosis and host cell death responses during Salmonella infection." Cell Microbiol **9**(11): 2562-70.
Finlay, B. B., F. Heffron, et al. (1989). "Epithelial cell surfaces induce Salmonella proteins required for bacterial adherence and invasion." Science **243**(4893): 940-3.
Flatley, J., J. Barrett, et al. (2005). "Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis." J Biol Chem **280**(11): 10065-72.

Fosse, J., H. Seegers, et al. (2008). "Foodborne zoonoses due to meat: a quantitative approach for a comparative risk assessment applied to pig slaughtering in Europe." Vet Res **39**(1): 1.
Foster, G., H. M. Ross, et al. (1998). "Salmonella typhimurium DT104 in a grey seal." Vet Rec **142**(22): 615.
Foster, J. W. (1991). "Salmonella acid shock proteins are required for the adaptive acid tolerance response." J Bacteriol **173**(21): 6896-902.
Foster, J. W. (2004). "Escherichia coli acid resistance: tales of an amateur acidophile." Nat Rev Microbiol **2**(11): 898-907.
Fournier, B., I. R. Williams, et al. (2009). "Toll-like receptor 5-dependent regulation of inflammation in systemic Salmonella enterica Serovar typhimurium infection." Infect Immun **77**(9): 4121-9.
Franchi, L., A. Amer, et al. (2006). "Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages." Nat Immunol **7**(6): 576-82.
Gahan, C. G. and C. Hill (1999). "The relationship between acid stress responses and virulence in Salmonella typhimurium and Listeria monocytogenes." Int J Food Microbiol **50**(1-2): 93-100.
Gahring, L. C., F. Heffron, et al. (1990). "Invasion and replication of Salmonella typhimurium in animal cells." Infect Immun **58**(2): 443-8.
Gaines, S., H. Sprinz, et al. (1968). "Studies on infection and immunity in experimental typhoid fever. VII. The distribution of Salmonella typhi in chimpanzee tissue following oral challenge, and the relationship between the numbers of bacilli and morphologic lesions." J Infect Dis **118**(3): 293-306.
Galan, J. E. (1996). "Molecular genetic bases of Salmonella entry into host cells." Mol Microbiol **20**(2): 263-71.
Galan, J. E. and R. Curtiss, 3rd (1989). "Virulence and vaccine potential of phoP mutants of Salmonella typhimurium." Microb Pathog **6**(6): 433-43.
Galan, J. E. and D. Zhou (2000). "Striking a balance: modulation of the actin cytoskeleton by Salmonella." Proc Natl Acad Sci U S A **97**(16): 8754-61.
Galanis, E., D. M. Lo Fo Wong, et al. (2006). "Web-based surveillance and global Salmonella distribution, 2000-2002." Emerg Infect Dis **12**(3): 381-8.
Galen, J. E., J. Nair, et al. (1999). "Optimization of plasmid maintenance in the attenuated live vector vaccine strain Salmonella typhi CVD 908-htrA." Infect Immun **67**(12): 6424-33.
Galen, J. E., M. F. Pasetti, et al. (2009). "Salmonella enterica serovar Typhi live vector vaccines finally come of age." Immunol Cell Biol **87**(5): 400-12.
Galkin, V. E., A. Orlova, et al. (2002). "The bacterial protein SipA polymerizes G-actin and mimics muscle nebulin." Nat Struct Biol **9**(7): 518-21.
Ganz, T. (2000). "Paneth cells--guardians of the gut cell hatchery." Nat Immunol **1**(2): 99-100.
Garcia-del Portillo, F. and B. B. Finlay (1994). "Invasion and intracellular proliferation of Salmonella within non-phagocytic cells." Microbiologia **10**(3): 229-38.
Garcia-del Portillo, F., M. B. Zwick, et al. (1993). "Intracellular replication of Salmonella within epithelial cells is associated with filamentous structures containing lysosomal membrane glycoproteins." Infect Agents Dis **2**(4): 227-31.
Gardner, A. M. and P. R. Gardner (2002). "Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, Escherichia coli. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity." J Biol Chem **277**(10): 8166-71.
Gardner, A. M., R. A. Helmick, et al. (2002). "Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in Escherichia coli." J Biol Chem **277**(10): 8172-7.
Gardner, P. R. (2005). "Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases." J Inorg Biochem **99**(1): 247-66.

Gardner, P. R., A. M. Gardner, et al. (1998). "Nitric oxide dioxygenase: an enzymic function for flavohemoglobin." Proc Natl Acad Sci U S A **95**(18): 10378-83.
Geddes, K., F. Cruz, et al. (2007). "Analysis of Cells Targeted by Salmonella Type III Secretion In Vivo." PLoS Pathog **3**(12): e196.
Gemski, P., Jr. and B. A. Stocker (1967). "Transduction by bacteriophage P22 in nonsmooth mutants of Salmonella typhimurium." J Bacteriol **93**(5): 1588-97.
Germanier, R. and E. Furer (1975). "Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine." J Infect Dis **131**(5): 553-8.
Gewirtz, A. T., A. S. Rao, et al. (2000). "Salmonella typhimurium induces epithelial IL-8 expression via Ca(2+)-mediated activation of the NF-kappaB pathway." J Clin Invest **105**(1): 79-92.
Gilberthorpe, N. J., M. E. Lee, et al. (2007). "NsrR: a key regulator circumventing Salmonella enterica serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages." Microbiology **153**(Pt 6): 1756-71.
Gilberthorpe, N. J. and R. K. Poole (2008). "Nitric oxide homeostasis in salmonella typhimurium: Roles of respiratory nitrate reductase and flavohemoglobin." J Biol Chem 283, 11146-11154. .
Glaser, P., A. Danchin, et al. (1995). "Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of Bacillus subtilis." J Bacteriol **177**(4): 1112-5.
Gobert, A. P., D. J. McGee, et al. (2001). "Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival." Proc Natl Acad Sci U S A **98**(24): 13844-9.
Goldstein, F. W., J. C. Chumpitaz, et al. (1986). "Plasmid-mediated resistance to multiple antibiotics in Salmonella typhi." J Infect Dis **153**(2): 261-6.
Gomes, C. M., A. Giuffre, et al. (2002). "A novel type of nitric-oxide reductase. Escherichia coli flavorubredoxin." J Biol Chem **277**(28): 25273-6.
Goossens, H., M. Ferech, et al. (2005). "Outpatient antibiotic use in Europe and association with resistance: a cross-national database study." Lancet **365**(9459): 579-87.
Gow, A. J. and J. S. Stamler (1998). "Reactions between nitric oxide and haemoglobin under physiological conditions." Nature **391**(6663): 169-73.
Green, J., B. Bennett, et al. (1996). "Reconstitution of the [4Fe-4S] cluster in FNR and demonstration of the aerobic-anaerobic transcription switch in vitro." Biochem J **316 (Pt 3)**: 887-92.
Green, S. J., C. A. Nacy, et al. (1991). "Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens." J Leukoc Biol **50**(1): 93-103.
Greenacre, E. J., S. Lucchini, et al. (2006). "The lactic acid-induced acid tolerance response in Salmonella enterica serovar Typhimurium induces sensitivity to hydrogen peroxide." Appl Environ Microbiol **72**(8): 5623-5.
Gremillon, D. H., R. Geckler, et al. (1977). "Salmonella abscess. A potential nosocomial hazard." Arch Surg **112**(7): 843-5.
Griffith, O. W. and D. J. Stuehr (1995). "Nitric oxide synthases: properties and catalytic mechanism." Annu Rev Physiol **57**: 707-36.
Gunsalus, R. P. and S. J. Park (1994). "Aerobic-anaerobic gene regulation in Escherichia coli: control by the ArcAB and Fnr regulons." Res Microbiol **145**(5-6): 437-50.
Hacker, J., G. Blum-Oehler, et al. (1997). "Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution." Mol Microbiol **23**(6): 1089-97.
Handsfield, H. H. and W. L. Whitlington (1996). "Antibiotic-resistant neisseria gonorrhoeae: the calm before another storm?" Ann Intern Med **125**(6): 507-9.

Hapfelmeier, S., K. Ehrbar, et al. (2004). "Role of the Salmonella pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in Salmonella enterica subspecies 1 serovar Typhimurium colitis in streptomycin-pretreated mice." Infect Immun **72**(2): 795-809.
Haque, A., F. Bowe, et al. (2004). "Early interactions of Salmonella enterica serovar typhimurium with human small intestinal epithelial explants." Gut **53**(10): 1424-30.
Haraga, A., M. B. Ohlson, et al. (2008). "Salmonellae interplay with host cells." Nat Rev Microbiol **6**(1): 53-66.
Hardt, W. D., L. M. Chen, et al. (1998). "S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells." Cell **93**(5): 815-26.
Hart, A. L., K. Lammers, et al. (2004). "Modulation of human dendritic cell phenotype and function by probiotic bacteria." Gut **53**(11): 1602-9.
Hausladen, A., A. J. Gow, et al. (1998). "Nitrosative stress: metabolic pathway involving the flavohemoglobin." Proc Natl Acad Sci U S A **95**(24): 14100-5.
Hautefort, I., A. Thompson, et al. (2007). "During infection of epithelial cells Salmonella enterica serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems." Cell Microbiol **10**(4): 958-984.
Hejfec, L. B. (1965). "Results of the Study of Typhoid Vaccines in Four Controlled Field Trials in the Ussr." Bull World Health Organ **32**: 1-14.
Helaine, S., J. A. Thompson, et al. (2010). "Dynamics of intracellular bacterial replication at the single cell level." Proc Natl Acad Sci U S A **107**(8): 3746-51.
Heller, K. B., E. C. Lin, et al. (1980). "Substrate specificity and transport properties of the glycerol facilitator of Escherichia coli." J Bacteriol **144**(1): 274-8.
Henard, C. A. a. V.-T., A., (2011). "Nitric Oxide and Salmonella Pathogenesis." Frontiers in Microbiology, Cellular and Infection Microbiology **2**(April 2011).
Hendriksen, R. S., M. Mikoleit, et al. (2009). "WHO Global Salm-Surv external quality assurance system for serotyping of Salmonella isolates from 2000 to 2007." J Clin Microbiol **47**(9): 2729-36.
Hensel, M. (2000). "Salmonella pathogenicity island 2." Mol Microbiol **36**(5): 1015-23.
Hernandez, L. D., M. Pypaert, et al. (2003). "A Salmonella protein causes macrophage cell death by inducing autophagy." J Cell Biol **163**(5): 1123-31.
Herold, B. C., L. C. Immergluck, et al. (1998). "Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk." JAMA **279**(8): 593-8.
Hersh, D., D. M. Monack, et al. (1999). "The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1." Proc Natl Acad Sci U S A **96**(5): 2396-401.
Hoelzer, K., Y. Soyer, et al. (2010). "The prevalence of multidrug resistance is higher among bovine than human Salmonella enterica serotype Newport, Typhimurium, and 4,5,12:i:- isolates in the United States but differs by serotype and geographic region." Appl Environ Microbiol **76**(17): 5947-59.
Hohmann, E. L., C. A. Oletta, et al. (1996). "Evaluation of a phoP/phoQ-deleted, aroA-deleted live oral Salmonella typhi vaccine strain in human volunteers." Vaccine **14**(1): 19-24.
Hoiseth, S. K. and B. A. Stocker (1981). "Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines." Nature **291**(5812): 238-9.
Holden, D. W. (2002). "Trafficking of the Salmonella vacuole in macrophages." Traffic **3**(3): 161-9.
Hollinger, K., C. Wray, et al. (1998). "Salmonella Typhimurium DT104 in cattle in Great Britain." J Am Vet Med Assoc **213**(12): 1732-3.
Hone, D. M., S. R. Attridge, et al. (1988). "A galE via (Vi antigen-negative) mutant of Salmonella typhi Ty2 retains virulence in humans." Infect Immun **56**(5): 1326-33.

Hone, D. M., A. M. Harris, et al. (1991). "Construction of genetically defined double aro mutants of Salmonella typhi." Vaccine **9**(11): 810-6.
Hook, E. W., 3rd, W. E. Brady, et al. (1989). "Determinants of emergence of antibiotic-resistant Neisseria gonorrhoeae." J Infect Dis **159**(5): 900-7.
Hoops, P., N. E. Prather, et al. (1976). "Evidence for an extrinsic immunogen in effective ribosomal vaccines from Salmonella typhimurium." Infect Immun **13**(4): 1184-92.
Hornick, R. B. (1970). "Pathogenesis of typhoid fever." J Egypt Public Health Assoc **45**(1): 247-59.
Hornick, R. B., S. E. Greisman, et al. (1970). "Typhoid fever: pathogenesis and immunologic control. 2." N Engl J Med **283**(14): 739-46.
Hosoglu, S., M. Aldemir, et al. (2004). "Risk factors for enteric perforation in patients with typhoid Fever." Am J Epidemiol **160**(1): 46-50.
Hosoglu, S., M. K. Celen, et al. (2006). "Risk factors for typhoid fever among adult patients in Diyarbakir, Turkey." Epidemiol Infect **134**(3): 612-6.
House, D., V. A. Ho, et al. (2008). "Antibodies to the Vi capsule of Salmonella Typhi in the serum of typhoid patients and healthy control subjects from a typhoid endemic region." J Infect Dev Ctries **2**(4): 308-12.
HPA. (2010). "Health Protection Agency." Health Protection Agency; Infectious Diseases, Epidemiology and Surveillance, *Salmonella*, Disease Statistics, from http://www.hpa.org.uk/.
Huang, D. B. and H. L. DuPont (2005). "Problem pathogens: extra-intestinal complications of Salmonella enterica serotype Typhi infection." Lancet Infect Dis **5**(6): 341-8.
Hueck, C. J. (1998). "Type III protein secretion systems in bacterial pathogens of animals and plants." Microbiol Mol Biol Rev **62**(2): 379-433.
Humphreys, S., A. Stevenson, et al. (1999). "The alternative sigma factor, sigmaE, is critically important for the virulence of Salmonella typhimurium." Infect Immun **67**(4): 1560-8.
Hutchings, M. I., N. Mandhana, et al. (2002). "The NorR protein of Escherichia coli activates expression of the flavorubredoxin gene norV in response to reactive nitrogen species." J Bacteriol **184**(16): 4640-3.
Ingledew, W. J. and R. K. Poole (1984). "The respiratory chains of Escherichia coli." Microbiol Rev **48**(3): 222-71.
Iobbi, C., C. L. Santini, et al. (1987). "Biochemical and immunological evidence for a second nitrate reductase in Escherichia coli K12." Eur J Biochem **168**(2): 451-9.
Iuchi, S. and E. C. Lin (1987). "The narL gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine N-oxide reductase operons in Escherichia coli." Proc Natl Acad Sci U S A **84**(11): 3901-5.
Ivanoff, B., M. M. Levine, et al. (1994). "Vaccination against typhoid fever: present status." Bull World Health Organ **72**(6): 957-71.
JAMA (1960). "Antibiotic Resistant Staphylococci." Journal of the American Medical Association **173**(5): 545.
Jepson, B. J., S. Mohan, et al. (2007). "Spectropotentiometric and structural analysis of the periplasmic nitrate reductase from Escherichia coli." J Biol Chem **282**(9): 6425-37.
Jepson, M. A. and M. A. Clark (2001). "The role of M cells in Salmonella infection." Microbes Infect **3**(14-15): 1183-90.
Jepson, M. A., B. Kenny, et al. (2001). "Role of sipA in the early stages of Salmonella typhimurium entry into epithelial cells." Cell Microbiol **3**(6): 417-26.
Jesenberger, V., K. J. Procyk, et al. (2000). "Salmonella-induced caspase-2 activation in macrophages: a novel mechanism in pathogen-mediated apoptosis." J Exp Med **192**(7): 1035-46.
Johansson, M. E., M. Phillipson, et al. (2008). "The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria." Proc Natl Acad Sci U S A **105**(39): 15064-9.

Johnston, C., D. A. Pegues, et al. (1996). "Transcriptional activation of Salmonella typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily." Mol Microbiol **22**(4): 715-27.
Jones, B. D., N. Ghori, et al. (1994). "Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches." J Exp Med **180**(1): 15-23.
Jones, D. E. and C. L. Bevins (1992). "Paneth cells of the human small intestine express an antimicrobial peptide gene." J Biol Chem **267**(32): 23216-25.
Jones, S. A., F. Z. Chowdhury, et al. (2007). "Respiration of Escherichia coli in the mouse intestine." Infect Immun **75**(10): 4891-9.
Jormakka, M., D. Richardson, et al. (2004). "Architecture of NarGH reveals a structural classification of Mo-bisMGD enzymes." Structure **12**(1): 95-104.
Justino, M. C., J. B. Vicente, et al. (2005). "New genes implicated in the protection of anaerobically grown Escherichia coli against nitric oxide." J Biol Chem **280**(4): 2636-43.
Kaback, H. R. (1968). "The role of the phosphoenolpyruvate-phosphotransferase system in the transport of sugars by isolated membrane preparations of Escherichia coli." J Biol Chem **243**(13): 3711-24.
Khan, M., Y. M. Coovadia, et al. (1999). "Influence of sex on clinical features, laboratory findings, and complications of typhoid fever." Am J Trop Med Hyg **61**(1): 41-6.
Khan, S. A., R. Stratford, et al. (2003). "Salmonella typhi and S typhimurium derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors." Vaccine **21**(5-6): 538-48.
Kim, S. O., Y. Orii, et al. (1999). "Anoxic function for the Escherichia coli flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide." FEBS Lett **445**(2-3): 389-94.
Kindt, T. J., Goldsby, R.A., Osborne, B.A., (2007). Kuby Immunology. New York, W.H. Freeman and Company.
King, T. and T. Ferenci (2005). "Divergent roles of RpoS in Escherichia coli under aerobic and anaerobic conditions." FEMS Microbiol Lett **244**(2): 323-7.
Klugman, K. P., I. T. Gilbertson, et al. (1987). "Protective activity of Vi capsular polysaccharide vaccine against typhoid fever." Lancet **2**(8569): 1165-9.
Klugman, K. P., H. J. Koornhof, et al. (1996). "Immunogenicity, efficacy and serological correlate of protection of Salmonella typhi Vi capsular polysaccharide vaccine three years after immunization." Vaccine **14**(5): 435-8.
Koch, A. L. (2007). "Evolution of temperate pathogens: the bacteriophage/bacteria paradigm." Virol J **4**: 121.
Kothari, S., Kothari, N., Lee, E., Kim, J.A., An, S.J., Yoon, Y.K., Smith, G.W., Carbis, R. (2010). "Development of an efficient and scalable method for processing and purification of Vi capsular polysaccharide." Procedia in Vaccinology **2**: 78-81.
Kusters, J. G., G. A. Mulders-Kremers, et al. (1993). "Effects of multiplicity of infection, bacterial protein synthesis, and growth phase on adhesion to and invasion of human cell lines by Salmonella typhimurium." Infect Immun **61**(12): 5013-20.
Lahiri, A., S. M. Eswarappa, et al. (2010). "Altering the balance between pathogen containing vacuoles and lysosomes: a lesson from Salmonella." Virulence **1**(4): 325-9.
Lander, K. P. and K. P. Gill (1980). "Experimental infection of the bovine udder with Campylobacter coli/jejuni." J Hyg (Lond) **84**(3): 421-8.
Lavigne, J. P., D. O'Callaghan, et al. (2005). "Requirement of MgtC for Brucella suis intramacrophage growth: a potential mechanism shared by Salmonella enterica and Mycobacterium tuberculosis for adaptation to a low-Mg2+ environment." Infect Immun **73**(5): 3160-3.
Lawrence, J. G. and H. Ochman (1998). "Molecular archaeology of the Escherichia coli genome." Proc Natl Acad Sci U S A **95**(16): 9413-7.

Lee, C. A. and S. Falkow (1990). "The ability of Salmonella to enter mammalian cells is affected by bacterial growth state." Proc Natl Acad Sci U S A **87**(11): 4304-8.
Lenski, R. E. and R. M. May (1994). "The evolution of virulence in parasites and pathogens: reconciliation between two competing hypotheses." J Theor Biol **169**(3): 253-65.
Levin, B. R., V. Perrot, et al. (2000). "Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria." Genetics **154**(3): 985-97.
Levine, M. M., C. Ferreccio, et al. (1987). "Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation." Lancet **1**(8541): 1049-52.
Levine, M. M., J. Galen, et al. (1996). "Attenuated Salmonella as live oral vaccines against typhoid fever and as live vectors." J Biotechnol **44**(1-3): 193-6.
Levings, R. S., D. Lightfoot, et al. (2005). "The genomic island SGI1, containing the multiple antibiotic resistance region of Salmonella enterica serovar Typhimurium DT104 or variants of it, is widely distributed in other S. enterica serovars." J Bacteriol **187**(13): 4401-9.
Levy, M. N., B. M. Koeppen, et al. (2005). Berne and Levy, Principles of Physiology, Elsevier Mosby.
Lin, J. T. and V. Stewart (1998). "Nitrate assimilation by bacteria." Adv Microb Physiol **39**: 1-30, 379.
Locksley, R. M., M. L. Cohen, et al. (1982). "Multiply antibiotic-resistant Staphylococcus aureus: introduction, transmission, and evolution of nosocomial infection." Ann Intern Med **97**(3): 317-24.
Low, J. C., G. Hopkins, et al. (1996). "Antibiotic resistant Salmonella typhimurium DT104 in cattle." Vet Rec **138**(26): 650-1.
Lowe, D. C., T. C. Savidge, et al. (1999). "Characterization of candidate live oral Salmonella typhi vaccine strains harboring defined mutations in aroA, aroC, and htrA." Infect Immun **67**(2): 700-7.
Lucas, R. L. and C. A. Lee (2001). "Roles of hilC and hilD in regulation of hilA expression in Salmonella enterica serovar Typhimurium." J Bacteriol **183**(9): 2733-45.
Lundberg, J. O., E. Weitzberg, et al. (2004). "Nitrate, bacteria and human health." Nat Rev Microbiol **2**(7): 593-602.
Lundberg, U., U. Vinatzer, et al. (1999). "Growth phase-regulated induction of Salmonella-induced macrophage apoptosis correlates with transient expression of SPI-1 genes." J Bacteriol **181**(11): 3433-7.
Lupp, C., M. L. Robertson, et al. (2007). "Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae." Cell Host Microbe **2**(2): 119-29.
MacFarlane, A. S., M. G. Schwacha, et al. (1999). "In vivo blockage of nitric oxide with aminoguanidine inhibits immunosuppression induced by an attenuated strain of Salmonella typhimurium, potentiates Salmonella infection, and inhibits macrophage and polymorphonuclear leukocyte influx into the spleen." Infect Immun **67**(2): 891-8.
Macpherson, A. J., M. M. Martinic, et al. (2002). "The functions of mucosal T cells in containing the indigenous commensal flora of the intestine." Cell Mol Life Sci **59**(12): 2088-96.
Magasanik, B. (1961). "Catabolite repression." Cold Spring Harb Symp Quant Biol **26**: 249-56.
Magee, J. T., E. L. Pritchard, et al. (1999). "Antibiotic prescribing and antibiotic resistance in community practice: retrospective study, 1996-8." BMJ **319**(7219): 1239-40.
Maggini, S., E. S. Wintergerst, et al. (2007). "Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses." Br J Nutr **98 Suppl 1**: S29-35.
Mahle, W. T. and M. M. Levine (1993). "Salmonella typhi infection in children younger than five years of age." Pediatr Infect Dis J **12**(8): 627-31.

Malik, A. S. (2002). "Complications of bacteriologically confirmed typhoid fever in children." J Trop Pediatr **48**(2): 102-8.
Malm, S., Y. Tiffert, et al. (2009). "The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of Mycobacterium tuberculosis." Microbiology **155**(Pt 4): 1332-9.
Mandalari, G., R. M. Faulks, et al. (2008). "Release of protein, lipid, and vitamin E from almond seeds during digestion." J Agric Food Chem **56**(9): 3409-16.
Mann, B. A. and J. M. Slauch (1997). "Transduction of low-copy number plasmids by bacteriophage P22." Genetics **146**(2): 447-56.
Marletta, M. A. (1994). "Nitric oxide synthase: aspects concerning structure and catalysis." Cell **78**(6): 927-30.
Marrie, T. J., E. V. Haldane, et al. (1981). "Susceptibility of anaerobic bacteria to nine antimicrobial agents and demonstration of decreased susceptibility of Clostridium perfringens to penicillin." Antimicrob Agents Chemother **19**(1): 51-5.
Maskell, D. J. (2005). Salmonella Infections; Clinical, Immunological and Molecular Aspects. Cambridge, Cambridge University Press.
Mastroeni, P., A. Vazquez-Torres, et al. (2000). "Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo." J Exp Med **192**(2): 237-48.
McClelland, M., K. E. Sanderson, et al. (2001). "Complete genome sequence of Salmonella enterica serovar Typhimurium LT2." Nature **413**(6858): 852-6.
McGhie, E. J., L. C. Brawn, et al. (2009). "Salmonella takes control: effector-driven manipulation of the host." Curr Opin Microbiol **12**(1): 117-24.
McKnight, G. M., L. M. Smith, et al. (1997). "Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans." Gut **40**(2): 211-4.
Meltzer, E. and E. Schwartz (2010). "Enteric fever: a travel medicine oriented view." Curr Opin Infect Dis **23**(5): 432-7.
Miao, E. A., C. M. Alpuche-Aranda, et al. (2006). "Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf." Nat Immunol **7**(6): 569-75.
Michel, T. and O. Feron (1997). "Nitric oxide synthases: which, where, how, and why?" J Clin Invest **100**(9): 2146-52.
Mills, P. C., G. Rowley, et al. (2008). "A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects Salmonella enterica serovar Typhimurium against killing by NO in anoxic environments." Microbiology **154**(Pt 4): 1218-28.
Mohan, S. B., Cole, J.A. (2007). The Dissimilatory Reduction of Nitrate to Ammonia by Anaerobic Bacteria. Biology of the Nitrogen Cycle. H. Bothe, Ferguson, S.J., Newton, W.E. . Netherlands, Elsevier**:** 93-107.
Monack, D. M., C. S. Detweiler, et al. (2001). "Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1." Cell Microbiol **3**(12): 825-37.
Monack, D. M., B. Raupach, et al. (1996). "Salmonella typhimurium invasion induces apoptosis in infected macrophages." Proc Natl Acad Sci U S A **93**(18): 9833-8.
Moncada, S., A. Higgs, et al. (1997). "International Union of Pharmacology Nomenclature in Nitric Oxide Research." Pharmacol Rev **49**(2): 137-42.
Monk, I. R., P. G. Casey, et al. (2008). "Development of multiple strain competitive index assays for Listeria monocytogenes using pIMC; a new site-specific integrative vector." BMC Microbiol **8**: 96.
Morgan, E., J. D. Campbell, et al. (2004). "Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium." Mol Microbiol **54**(4): 994-1010.
Morgenstern, R. and P. C. Hayes (1991). "The liver in typhoid fever: always affected, not just a complication." Am J Gastroenterol **86**(9): 1235-9.

Mukhopadhyay, P., M. Zheng, et al. (2004). "Prominent roles of the NorR and Fur regulators in the Escherichia coli transcriptional response to reactive nitrogen species." Proc Natl Acad Sci U S A **101**(3): 745-50.
Mumy, K. L. and B. A. McCormick (2005). "Events at the host-microbial interface of the gastrointestinal tract. II. Role of the intestinal epithelium in pathogen-induced inflammation." Am J Physiol Gastrointest Liver Physiol **288**(5): G854-9.
Murphy, C., C. Carroll, et al. (2005). "The effect of different media on the survival and induction of stress responses by Campylobacter jejuni." J Microbiol Methods **62**(2): 161-6.
Murray, B. E. (1994). "Can antibiotic resistance be controlled?" N Engl J Med **330**(17): 1229-30.
Nathan, C. and Q. W. Xie (1994). "Nitric oxide synthases: roles, tolls, and controls." Cell **78**(6): 915-8.
Navarre, W. W. and A. Zychlinsky (2000). "Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies." Cell Microbiol **2**(4): 265-73.
Neidhardt, F. C. (1996). Escherichia coli and Salmonella cellular and molecular biology. Washington D.C., ASM Press.
Nelson, D. L. and M. M. Cox (2004). Lehninger, Principles of Biochemistry, W.H.Freeman and Co.
Neva, F. A. (1950). "Pulmonary involvement in typhoid and paratyphoid fevers." Ann Intern Med **33**(1): 83-99.
Niedergang, F., A. Didierlaurent, et al. (2004). "Dendritic cells: the host Achille's heel for mucosal pathogens?" Trends Microbiol **12**(2): 79-88.
Niess, J. H., S. Brand, et al. (2005). "CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance." Science **307**(5707): 254-8.
Nnalue, N. A., A. Shnyra, et al. (1992). "Salmonella choleraesuis and Salmonella typhimurium associated with liver cells after intravenous inoculation of rats are localized mainly in Kupffer cells and multiply intracellularly." Infect Immun **60**(7): 2758-68.
Nsutebu, E. F., P. Martins, et al. (2003). "Prevalence of typhoid fever in febrile patients with symptoms clinically compatible with typhoid fever in Cameroon." Trop Med Int Health **8**(6): 575-8.
Ochman, H. and E. A. Groisman (1996). "Distribution of pathogenicity islands in Salmonella spp." Infect Immun **64**(12): 5410-2.
Ochman, H., F. C. Soncini, et al. (1996). "Identification of a pathogenicity island required for Salmonella survival in host cells." Proc Natl Acad Sci U S A **93**(15): 7800-4.
Okuma, K., K. Iwakawa, et al. (2002). "Dissemination of new methicillin-resistant Staphylococcus aureus clones in the community." J Clin Microbiol **40**(11): 4289-94.
Oosterom, J. (1991). "Epidemiological studies and proposed preventive measures in the fight against human salmonellosis." Int J Food Microbiol **12**(1): 41-51.
Owen, R. L. (1994). "M cells--entryways of opportunity for enteropathogens." J Exp Med **180**(1): 7-9.
Owen, R. L. and T. H. Ermak (1990). "Structural specializations for antigen uptake and processing in the digestive tract." Springer Semin Immunopathol **12**(2-3): 139-52.
Owen, R. L. and A. L. Jones (1974). "Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles." Gastroenterology **66**(2): 189-203.
Pace, J., M. J. Hayman, et al. (1993). "Signal transduction and invasion of epithelial cells by S. typhimurium." Cell **72**(4): 505-14.
Palmer, K. L., S. A. Brown, et al. (2007). "Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum." J Bacteriol **189**(12): 4449-55.
Pang, T. and S. D. Puthucheary (1983). "Significance and value of the Widal test in the diagnosis of typhoid fever in an endemic area." J Clin Pathol **36**(4): 471-5.

Parkhill, J., G. Dougan, et al. (2001). "Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18." Nature **413**(6858): 848-52.
Parry, C., J. Wain, et al. (1998). "Quinolone-resistant Salmonella typhi in Vietnam." Lancet **351**(9111): 1289.
Parry, C. M., T. T. Hien, et al. (2002). "Typhoid fever." N Engl J Med **347**(22): 1770-82.
Parry, C. M., N. T. Hoa, et al. (1999). "Value of a single-tube widal test in diagnosis of typhoid fever in Vietnam." J Clin Microbiol **37**(9): 2882-6.
Pasetti, M. F., M. M. Levine, et al. (2003). "Animal models paving the way for clinical trials of attenuated Salmonella enterica serovar Typhi live oral vaccines and live vectors." Vaccine **21**(5-6): 401-18.
Pawelek, J. M., K. B. Low, et al. (1997). "Tumor-targeted Salmonella as a novel anticancer vector." Cancer Res **57**(20): 4537-44.
Peeters, T. and G. Vantrappen (1975). "The Paneth cell: a source of intestinal lysozyme." Gut **16**(7): 553-8.
Perrett, C. A. and M. A. Jepson (2009). "Regulation of Salmonella-induced membrane ruffling by SipA differs in strains lacking other effectors." Cell Microbiol **11**(3): 475-87.
Pesci, E. C., D. L. Cottle, et al. (1994). "Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of Campylobacter jejuni." Infect Immun **62**(7): 2687-94.
Philpott, D. J. and S. E. Girardin (2004). "The role of Toll-like receptors and Nod proteins in bacterial infection." Mol Immunol **41**(11): 1099-108.
Pittman, M. S., K. T. Elvers, et al. (2007). "Growth of Campylobacter jejuni on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress." Mol Microbiol **63**(2): 575-90.
Poock, S. R., E. R. Leach, et al. (2002). "Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of Escherichia coli." J Biol Chem **277**(26): 23664-9.
Poole, R. K., M. F. Anjum, et al. (1996). "Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12." J Bacteriol **178**(18): 5487-92.
Popoff, M. Y., J. Bockemuhl, et al. (2003). "Supplement 2001 (no. 45) to the Kauffmann-White scheme." Res Microbiol **154**(3): 173-4.
Popoff, M. Y., J. Bockemuhl, et al. (2004). "Supplement 2002 (no. 46) to the Kauffmann-White scheme." Res Microbiol **155**(7): 568-70.
Porter, E. M., C. L. Bevins, et al. (2002). "The multifaceted Paneth cell." Cell Mol Life Sci **59**(1): 156-70.
Postma, P. W., O. M. Neyssel, et al. (1982). "Glucose transport in Salmonella typhimurium and Escherichia coli." Eur J Biochem **123**(1): 113-9.
Potter, L. C. and J. A. Cole (1999). "Essential roles for the products of the napABCD genes, but not napFGH, in periplasmic nitrate reduction by Escherichia coli K-12." Biochem J **344 Pt 1**: 69-76.
Potter, L. C., P. Millington, et al. (1999). "Competition between Escherichia coli strains expressing either a periplasmic or a membrane-bound nitrate reductase: does Nap confer a selective advantage during nitrate-limited growth?" Biochem J **344 Pt 1**: 77-84.
Pullan, S. T., M. D. Gidley, et al. (2007). "Nitric oxide in chemostat-cultured Escherichia coli is sensed by Fnr and other global regulators: unaltered methionine biosynthesis indicates lack of S nitrosation." J Bacteriol **189**(5): 1845-55.
Rahman, S. H., B. J. Ammori, et al. (2003). "Increased nitric oxide excretion in patients with severe acute pancreatitis: evidence of an endotoxin mediated inflammatory response?" Gut **52**(2): 270-4.
Raines, K. W., T. J. Kang, et al. (2006). "Importance of nitric oxide synthase in the control of infection by Bacillus anthracis." Infect Immun **74**(4): 2268-76.
Rajashekara, G., E. Haverly, et al. (2000). "Multidrug-resistant Salmonella Typhimurium DT104 in poultry." J Food Prot **63**(2): 155-61.

Rappl, C., J. Deiwick, et al. (2003). "Acidic pH is required for the functional assembly of the type III secretion system encoded by Salmonella pathogenicity island 2." FEMS Microbiol Lett **226**(2): 363-72.
Rath, H. C., K. H. Wilson, et al. (1999). "Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with Bacteroides vulgatus or Escherichia coli." Infect Immun **67**(6): 2969-74.
Rathman, M., M. D. Sjaastad, et al. (1996). "Acidification of phagosomes containing Salmonella typhimurium in murine macrophages." Infect Immun **64**(7): 2765-73.
Rayan, F., C. Mukundan, et al. (2009). "A case of relapsing Salmonella osteomyelitis in a thalassaemia trait patient." J Orthop Traumatol **10**(1): 31-3.
Rescigno, M., G. Rotta, et al. (2001). "Dendritic cells shuttle microbes across gut epithelial monolayers." Immunobiology **204**(5): 572-81.
Resta-Lenert, S., J. Smitham, et al. (2005). "Epithelial dysfunction associated with the development of colitis in conventionally housed mdr1a-/- mice." Am J Physiol Gastrointest Liver Physiol **289**(1): G153-62.
Ridley, M., R. Lynn, et al. (1970). "Antibiotic-resistant Staphylococcus aureus and hospital antibiotic policies." Lancet **1**(7640): 230-3.
Roark, E. A. and K. Haldar (2008). "Effects of lysosomal membrane protein depletion on the Salmonella-containing vacuole." PLoS One **3**(10): e3538.
Robbe-Saule, V., C. Coynault, et al. (1995). "The live oral typhoid vaccine Ty21a is a rpoS mutant and is susceptible to various environmental stresses." FEMS Microbiol Lett **126**(2): 171-6.
Robbins, J. D. and J. B. Robbins (1984). "Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of Salmonella typhi." J Infect Dis **150**(3): 436-49.
Roberts, M., J. Li, et al. (1998). "Oral vaccination against tetanus: comparison of the immunogenicities of Salmonella strains expressing fragment C from the nirB and htrA promoters." Infect Immun **66**(7): 3080-7.
Rogler, G., K. Brand, et al. (1998). "Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa." Gastroenterology **115**(2): 357-69.
Roos, D. and C. C. Winterbourn (2002). "Immunology. Lethal weapons." Science **296**(5568): 669-71.
Roumagnac, P., F. X. Weill, et al. (2006). "Evolutionary history of Salmonella typhi." Science **314**(5803): 1301-4.
Rowley, G., D. Hensen, et al. (2012). "Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in Salmonella enterica serovar Typhimurium." Biochem J **441** 755 - 762.
Roy, M. F., L. Lariviere, et al. (2006). "Incremental expression of Tlr4 correlates with mouse resistance to Salmonella infection and fine regulation of relevant immune genes." Genes Immun **7**(5): 372-83.
Rugbjerg, H., A. Wingstrand, et al. (2004). "Estimating the number of undetected multi-resistant Salmonella Typhimurium DT104 infected pig herds in Denmark." Prev Vet Med **65**(3-4): 147-71.
Rychlik, I. and P. A. Barrow (2005). "Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection." FEMS Microbiol Rev **29**(5): 1021-40.
Salyers, A. A. and C. F. Amabile-Cuevas (1997). "Why are antibiotic resistance genes so resistant to elimination?" Antimicrob Agents Chemother **41**(11): 2321-5.
Sambrook, J. and D. Russell (2001). Molecular Cloning: A Laboratory Manual (Third Edition), Cold Spring Harbor Laboratory Press.
Sansonetti, P. J. (2004). "War and peace at mucosal surfaces." Nat Rev Immunol **4**(12): 953-64.

Sansonetti, P. J., A. Phalipon, et al. (2000). "Caspase-1 activation of IL-1beta and IL-18 are essential for Shigella flexneri-induced inflammation." Immunity **12**(5): 581-90.
Sanyal, D., T. Douglas, et al. (1997). "Salmonella infection acquired from reptilian pets." Arch Dis Child **77**(4): 345-6.
Saunders, J. R. (1984). "Genetics and evolution of antibiotic resistance." Br Med Bull **40**(1): 54-60.
Schechter, L. M., S. Jain, et al. (2003). "The small nucleoid-binding proteins H-NS, HU, and Fis affect hilA expression in Salmonella enterica serovar Typhimurium." Infect Immun **71**(9): 5432-5.
Scherer, C. A., & Miller, S.I. (2001). Molecular Pathogenesis of Salmonellae. Principles of Bacterial Pathogenesis. E. A. Groisman. San Diego, California, Academic Press**:** 265-335.
Schmieger, H. (1972). "Phage P22-mutants with increased or decreased transduction abilities." Mol Gen Genet **119**(1): 75-88.
Schneider, R., A. Travers, et al. (1997). "FIS modulates growth phase-dependent topological transitions of DNA in Escherichia coli." Mol Microbiol **26**(3): 519-30.
Schreiber, K., R. Krieger, et al. (2007). "The anaerobic regulatory network required for Pseudomonas aeruginosa nitrate respiration." J Bacteriol **189**(11): 4310-4.
Schroeder, S. A., P. M. Terry, et al. (1968). "Antibiotic resistance and transfer factor in Salmonella, United States 1967." JAMA **205**(13): 903-6.
Sears, H. J., B. Bennett, et al. (1995). "Identification of periplasmic nitrate reductase Mo(V) EPR signals in intact cells of Paracoccus denitrificans." Biochem J **310 (Pt 1)**: 311-4.
Sears, S. D., C. Ferreccio, et al. (1984). "The use of Moore swabs for isolation of Salmonella typhi from irrigation water in Santiago, Chile." J Infect Dis **149**(4): 640-2.
Selander, R. K., P. Beltran, et al. (1990). "Evolutionary genetic relationships of clones of Salmonella serovars that cause human typhoid and other enteric fevers." Infect Immun **58**(7): 2262-75.
Senewiratne, B. and K. Senewiratne (1977). "Reassessment of the Widal test in the diagnosis of typhoid." Gastroenterology **73**(2): 233-6.
Shea, J. E., M. Hensel, et al. (1996). "Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium." Proc Natl Acad Sci U S A **93**(6): 2593-7.
Shiloh, M. U., J. D. MacMicking, et al. (1999). "Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase." Immunity **10**(1): 29-38.
Silva, B. A., C. Gonzalez, et al. (1987). "Genetic characteristics of the Salmonella typhi strain Ty21a vaccine." J Infect Dis **155**(5): 1077-8.
Simanjuntak, C. H., F. P. Paleologo, et al. (1991). "Oral immunisation against typhoid fever in Indonesia with Ty21a vaccine." Lancet **338**(8774): 1055-9.
Simon, J., M. Sanger, et al. (2003). "Electron transport to periplasmic nitrate reductase (NapA) of Wolinella succinogenes is independent of a NapC protein." Mol Microbiol **49**(1): 69-79.
Simpson, P. J., D. J. Richardson, et al. (2010). "The periplasmic nitrate reductase in Shewanella: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB." Microbiology **156**(Pt 2): 302-12.
Smith, J. L. (2003). "The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions." J Food Prot **66**(7): 1292-303.
Sodergren, E. J., P. Y. Hsu, et al. (1988). "Roles of the narJ and narI gene products in the expression of nitrate reductase in Escherichia coli." J Biol Chem **263**(31): 16156-62.
Sohaskey, C. D. (2008). "Nitrate enhances the survival of Mycobacterium tuberculosis during inhibition of respiration." J Bacteriol **190**(8): 2981-6.

Sohaskey, C. D. and L. G. Wayne (2003). "Role of narK2X and narGHJI in hypoxic upregulation of nitrate reduction by Mycobacterium tuberculosis." J Bacteriol **185**(24): 7247-56.
Sojka, W. J. and E. B. Hudson (1976). "A survey of drug resistance in Salmonella isolated from animals in England and Wales during 1972." Br Vet J **132**(1): 95-104.
Sorensen, S. J., M. Bailey, et al. (2005). "Studying plasmid horizontal transfer in situ: a critical review." Nat Rev Microbiol **3**(9): 700-10.
Spaink, H. P., Okker, R.J.H., Wijffelman, C.A., Pees, E., Lugtenberg, B.J.J., (1987). "Promoters in the nodulation region of the Rhizobium leguminosarum symbiotic plasmid pRL1JI." Plant Molecular Biology **9**: 27-39.
Spector, M. P., F. Garcia del Portillo, et al. (1999). "The rpoS-dependent starvation-stress response locus stiA encodes a nitrate reductase (narZYWV) required for carbon-starvation-inducible thermotolerance and acid tolerance in Salmonella typhimurium." Microbiology **145 (Pt 11)**: 3035-45.
Spiro, S., K. L. Gaston, et al. (1990). "Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR." Mol Microbiol **4**(11): 1831-8.
Staats, H. F., R. J. Jackson, et al. (1994). "Mucosal immunity to infection with implications for vaccine development." Curr Opin Immunol **6**(4): 572-83.
Staats, H. F., S. M. Kirwan, et al. (2010). "Development of a bead immunoassay to measure Vi polysaccharide-specific serum IgG after vaccination with the Salmonella enterica serovar Typhi Vi polysaccharide." Clin Vaccine Immunol **17**(3): 412-9.
Stecher, B., M. Barthel, et al. (2008). "Motility allows S. Typhimurium to benefit from the mucosal defence." Cell Microbiol **10**(5): 1166-80.
Stecher, B. and W. D. Hardt (2008). "The role of microbiota in infectious disease." Trends Microbiol **16**(3): 107-14.
Stecher, B., R. Robbiani, et al. (2007). "Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota." PLoS Biol **5**(10): 2177-89.
Steele-Mortimer, O., S. Meresse, et al. (1999). "Biogenesis of Salmonella typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway." Cell Microbiol **1**(1): 33-49.
Stenuit, B., L. Eyers, et al. (2006). "Aerobic growth of Escherichia coli with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source and evidence of TNT denitration by whole cells and cell-free extracts." Appl Environ Microbiol **72**(12): 7945-8.
Stephens, J. C., M. J. Darsley, et al. (2006). "Stabilization of a plasmid coding for a heterologous antigen in Salmonella enterica serotype typhi vaccine strain CVD908-htrA by using site-specific recombination." Infect Immun **74**(7): 4383-6.
Stevanin, T. M., R. K. Poole, et al. (2002). "Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages." Infect Immun **70**(8): 4399-405.
Stevanin, T. M., R. C. Read, et al. (2007). "The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility." Gene **398**(1-2): 62-8.
Stewart, V. (1982). "Requirement of Fnr and NarL functions for nitrate reductase expression in Escherichia coli K-12." J Bacteriol **151**(3): 1320-5.
Stewart, V. (1988). "Nitrate respiration in relation to facultative metabolism in enterobacteria." Microbiol Rev **52**(2): 190-232.
Stewart, V. (1993). "Nitrate regulation of anaerobic respiratory gene expression in Escherichia coli." Mol Microbiol **9**(3): 425-34.
Stewart, V. (1994). "Regulation of nitrate and nitrite reductase synthesis in enterobacteria." Antonie Van Leeuwenhoek **66**(1-3): 37-45.
Stewart, V. and B. L. Berg (1988). "Influence of nar (nitrate reductase) genes on nitrate inhibition of formate-hydrogen lyase and fumarate reductase gene expression in Escherichia coli K-12." J Bacteriol **170**(10): 4437-44.

Stewart, V., P. J. Bledsoe, et al. (2009). "Catabolite repression control of napF (periplasmic nitrate reductase) operon expression in Escherichia coli K-12." J Bacteriol **191**(3): 996-1005.
Stewart, V., Y. Lu, et al. (2002). "Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by Escherichia coli K-12." J Bacteriol **184**(5): 1314-23.
Stickland, L. H. (1934). "Studies in the metabolism of the strict anaerobes (genus Clostridium): The chemical reactions by which Cl. sporogenes obtains its energy." Biochem J **28**(5): 1746-59.
Stoleru, G. H., L. Le Minor, et al. (1976). "Polynucleotide sequence divergence among strains of Salmonella sub-genus IV and closely related organisms." Ann Microbiol (Paris) **127**(4): 477-86.
Tacket, C. O., M. B. Sztein, et al. (1997). "Safety of live oral Salmonella typhi vaccine strains with deletions in htrA and aroC aroD and immune response in humans." Infect Immun **65**(2): 452-6.
Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.
Takeuchi, O., K. Hoshino, et al. (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components." Immunity **11**(4): 443-51.
Tam, C. C., S. J. O'Brien, et al. (2003). "Campylobacter coli - an important foodborne pathogen." J Infect **47**(1): 28-32.
Tapa, S. and B. Cvjetanovic (1975). "Controlled field trial on the effectiveness of one and two doses of acetone-inactivated and dried typhoid vaccine." Bull World Health Organ **52**(1): 75-80.
Teixido, L., P. Cortes, et al. (2010). "Control by Fur of the nitrate respiration regulators NarP and NarL in Salmonella enterica." Int Microbiol **13**(1): 33-9.
Telenti, A., P. Imboden, et al. (1993). "Detection of rifampicin-resistance mutations in Mycobacterium tuberculosis." Lancet **341**(8846): 647-50.
Ten Bruggencate, S. J., I. M. Bovee-Oudenhoven, et al. (2004). "Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium." Gut **53**(4): 530-5.
Thompson, A., G. Rowley, et al. (2006). "Salmonella transcriptomics: relating regulons, stimulons and regulatory networks to the process of infection." Curr Opin Microbiol **9**(1): 109-16.
Thompson, J. F., L. Moitoso de Vargas, et al. (1987). "Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway." Cell **50**(6): 901-8.
Threlfall, E. J. (2002). "Antimicrobial drug resistance in Salmonella: problems and perspectives in food- and water-borne infections." FEMS Microbiol Rev **26**(2): 141-8.
Threlfall, E. J., L. R. Ward, et al. (1997). "Increase in multiple antibiotic resistance in nontyphoidal salmonellas from humans in England and Wales: a comparison of data for 1994 and 1996." Microb Drug Resist **3**(3): 263-6.
Tomasz, A. and S. Waks (1975). "Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis." Proc Natl Acad Sci U S A **72**(10): 4162-6.
Troxell, B., R. C. Fink, et al. (2011). "The Fur regulon in anaerobically grown Salmonella enterica sv. Typhimurium: identification of new Fur targets." BMC Microbiol **11**: 236.
Troxell, B., M. L. Sikes, et al. (2010). "Fur negatively regulates hns and is required for the expression of HilA and virulence in Salmonella enterica serovar Typhimurium." J Bacteriol **193**(2): 497-505.

Tucker, N. P., B. D'Autreaux, et al. (2004). "DNA binding activity of the Escherichia coli nitric oxide sensor NorR suggests a conserved target sequence in diverse proteobacteria." J Bacteriol **186**(19): 6656-60.
Tucker, N. P., M. G. Hicks, et al. (2008). "The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster." PLoS One **3**(11): e3623.
Tukel, C., M. Raffatellu, et al. (2006). "Neutrophil influx during non-typhoidal salmonellosis: who is in the driver's seat?" FEMS Immunol Med Microbiol **46**(3): 320-9.
Umezawa, K., T. Akaike, et al. (1997). "Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against Salmonella typhimurium infection in mice." Infect Immun **65**(7): 2932-40.
Van Alst, N. E., K. F. Picardo, et al. (2007). "Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in Pseudomonas aeruginosa." Infect Immun **75**(8): 3780-90.
van der Velden, A. W., S. W. Lindgren, et al. (2000). "Salmonella pathogenicity island 1-independent induction of apoptosis in infected macrophages by Salmonella enterica serotype typhimurium." Infect Immun **68**(10): 5702-9.
van Wonderen, J. H., B. Burlat, et al. (2008). "The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli." J Biol Chem.**283** (15):9587-94
Vazquez-Torres, A., J. Jones-Carson, et al. (2000). "Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro." J Exp Med **192**(2): 227-36.
Vazquez-Torres, A., Y. Xu, et al. (2000). "Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase." Science **287**(5458): 1655-8.
Velge, P., A. Cloeckaert, et al. (2005). "Emergence of Salmonella epidemics: the problems related to Salmonella enterica serotype Enteritidis and multiple antibiotic resistance in other major serotypes." Vet Res **36**(3): 267-88.
Wall, P. G., D. Morgan, et al. (1994). "A case control study of infection with an epidemic strain of multiresistant Salmonella typhimurium DT104 in England and Wales." Commun Dis Rep CDR Rev **4**(11): R130-5.
Wall, P. G., E. J. Threlfall, et al. (1996). "Multiresistant Salmonella typhimurium DT104 in cats: a public health risk." Lancet **348**(9025): 471.
Wallis, T. S. and E. E. Galyov (2000). "Molecular basis of Salmonella-induced enteritis." Mol Microbiol **36**(5): 997-1005.
Wang, B. (2001). "Limitations of compositional approach to identifying horizontally transferred genes." J Mol Evol **53**(3): 244-50.
Wang, F., X. J. Gu, et al. (1989). "Treatment of typhoid fever with ofloxacin." J Antimicrob Chemother **23**(5): 785-8.
Wang, H. and R. P. Gunsalus (2000). "The nrfA and nirB nitrite reductase operons in Escherichia coli are expressed differently in response to nitrate than to nitrite." J Bacteriol **182**(20): 5813-22.
Wassef, J. S., D. F. Keren, et al. (1989). "Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis." Infect Immun **57**(3): 858-63.
Weber, I., C. Fritz, et al. (2000). "Anaerobic nitrate reductase (narGHJI) activity of Mycobacterium bovis BCG in vitro and its contribution to virulence in immunodeficient mice." Mol Microbiol **35**(5): 1017-25.
Weller, R., S. Pattullo, et al. (1996). "Nitric oxide is generated on the skin surface by reduction of sweat nitrate." J Invest Dermatol **107**(3): 327-31.
WHO. (2002). "Factsheet No. 194 - Antimicrobial Resistance ", from http://www.who.int/mediacentre/factsheets/fs194/en/.

WHO. (2009, February 2009). "Initiative for Vaccine Research - Diarrhoeal Diseases - Typhoid Fever ", from http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index7.html.

Wilson, R. L., S. J. Libby, et al. (2001). "Fis, a DNA nucleoid-associated protein, is involved in Salmonella typhimurium SPI-1 invasion gene expression." Mol Microbiol **39**(1): 79-88.

Wink, D. A., K. S. Kasprzak, et al. (1991). "DNA deaminating ability and genotoxicity of nitric oxide and its progenitors." Science **254**(5034): 1001-3.

Winter, S. E., P. Thiennimitr, et al. (2010). "Gut inflammation provides a respiratory electron acceptor for Salmonella." Nature **467**(7314): 426-9.

Winterbourn, C. C., M. B. Hampton, et al. (2006). "Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing." J Biol Chem **281**(52): 39860-9.

Wise, E. M., Jr. and J. T. Park (1965). "Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis." Proc Natl Acad Sci U S A **54**(1): 75-81.

Wray, C. and W. J. Sojka (1984). "The serological responses of calves to live Salmonella dublin vaccine--a comparison of different serological tests." J Biol Stand **12**(3): 277-82.

Xu, J., X. Xu, et al. (2001). "The bactericidal effect and chemical reactions of acidified nitrite under conditions simulating the stomach." J Appl Microbiol **90**(4): 523-9.

Xu, X. and M. Hensel (2010). "Systematic analysis of the SsrAB virulon of Salmonella enterica." Infect Immun **78**(1): 49-58.

Yamamoto, N. and M. L. Droffner (1985). "Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe Salmonella typhimurium." Proc Natl Acad Sci U S A **82**(7): 2077-81.

Zhang, S., R. A. Kingsley, et al. (2003). "Molecular pathogenesis of Salmonella enterica serotype typhimurium-induced diarrhea." Infect Immun **71**(1): 1-12.

Zoetendal, E. G., E. E. Vaughan, et al. (2006). "A microbial world within us." Mol Microbiol **59**(6): 1639-50.

Zychlinsky, A. and P. J. Sansonetti (1997). "Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death?" Trends Microbiol **5**(5): 201-4.